RESEARCH

Tetrac downregulates β -catenin and HMGA2 to promote the effect of resveratrol in colon cancer

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Abstract

The molecular pathogenesis of colorectal cancer encompasses the activation of several oncogenic signaling pathways that include the Wnt/ β -catenin pathway and the overexpression of high mobility group protein A2 (HMGA2). Resveratrol – the polyphenolic phytoalexin – binds to integrin $\alpha v \beta 3$ to induce apoptosis in cancer cells via cyclooxygenase 2 (COX-2) nuclear accumulation and p53-dependent apoptosis. Tetraiodothyroacetic acid (tetrac) is a de-aminated derivative of L-thyroxine (T_4), which – in contrast to the parental hormone – impairs cancer cell proliferation. In the current study, we found that tetrac promoted resveratrol-induced anti-proliferation in colon cancer cell lines, in primary cultures of colon cancer cells, and *in vivo*. The mechanisms implicated in this action involved the downregulation of nuclear β -catenin and HMGA2, which are capable of compromising resveratrol-induced COX-2 nuclear translocation. Silencing of either β -catenin or HMGA2 promoted resveratrol-induced anti-proliferation and COX-2 nuclear accumulation which is essential for integrin $\alpha v \beta 3$ -mediated-resveratrol-induced apoptosis in cancer cells. Concurrently, tetrac enhanced nuclear abundance of chibby family member 1, the nuclear β -catenin antagonist, which may further compromise the nuclear β -catenin-dependent gene expression and proliferation. Taken together, these results suggest that tetrac targets β -catenin and HMGA2 to promote resveratrol-induced-anti-proliferation in colon cancers, highlighting its potential in anti-cancer combination therapy.

Key Words

- ▶ tetraiodothyroacetic acid
- ▶ resveratrol
- ▶ β -catenin
- ▶ HMGA2
- ▶ colorectal cancers

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Introduction

Colorectal cancer is a worldwide burden, ranked as the third most prevalent and the fourth most lethal of all malignant neoplasms (Torre *et al.* 2015). The clinical management of this cancer is hindered by the substantial rate of relapse, which often leads to patients' death. Novel therapeutic approaches are needed for this disease. The molecular pathogenesis of colon cancer encompasses the activation of several oncogenic signaling pathways, including the Wnt/ β -catenin pathway and the overexpression of the high mobility group protein A2-hook 2 (HMGA2) (Morin *et al.* 1997, Li *et al.* 2014).

The Wnt/ β -catenin pathway is an evolutionarily-conserved cell signaling system that mediates key physiological processes, but is also incriminated in the occurrence of several malignant neoplasms, including colon cancer. The activation of this pathway leads to the nuclear translocation of β -catenin, the downstream transcription factor. Nuclear β -catenin forms a transcriptional complex with co-activator members of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors, to activate its target genes which are largely involved in cancer cell proliferation and metastasis (Clevers & Nusse 2012). Within the nucleus, β -catenin's activity is modulated by co-repressors. One such co-repressor is chibby family member 1, the gene product of *CBY1*, which antagonizes LEF at the promoter of β -catenin target genes (Takemaru *et al.* 2003).

HMGA2 is a non-histone chromatin-binding protein, which is abundantly expressed during embryogenesis, but barely detectable in normal adult tissues (Fusco & Fedele 2007, Xi *et al.* 2016). High levels of HMGA2 have been consistently associated with the development and progression of many malignant neoplasms, both in preclinical and clinical studies (Fusco & Fedele 2007, Wang *et al.* 2011, Günther *et al.* 2017). Accordingly, abundant expression of HMGA2 is correlated with metastasis and poor survival in colon cancer patients (Wang *et al.* 2011). Signaling via the phosphoinositide 3-kinase (PI3K) pathway, Ras/mitogen-activated protein kinase (MAPK) and oxidative stress has been implicated in vigorous activation of the *HMGA2* gene (Ayoubi *et al.* 1999, Zentner *et al.* 2001).

Tetraiodothyroacetic acid (tetrac) is a de-aminated analog of the thyroid hormone L-thyroxine (T_4). Tetrac has been shown to inhibit cancer cell proliferation *in vitro* (Lin *et al.* 2011a, Mousa *et al.* 2012, Cohen *et al.* 2018) and in animal xenograft models (Yalcin *et al.* 2009). At its receptor on the extracellular domain of plasma membrane

integrin $\alpha\beta_3$, tetrac blocks cancer cell proliferation induced by thyroid hormones (T_4 and T_3) (Schmohl *et al.* 2015). Importantly, T_4 has been shown to promote the nuclear accumulation of HMGA2 and β -catenin in a concentration-dependent manner in colorectal cancer cells with different *k*-RAS statuses (Lin 2016). However, independently of thyroid hormones, tetrac is capable of modulating the expression of genes regulated by cancer cells, which are important to cancer cell survival, angiogenesis, export of chemotherapeutic agents from cancer cells and repair of double-strand DNA breaks (Davis *et al.* 2014). Among such tetrac-regulated genes is *CBY1*, whose gene product is chibby family member 1, the nuclear β -catenin antagonist mentioned above (Glinskii *et al.* 2009, Lin *et al.* 2016b). Additionally, tetrac enhances the therapeutic potential of other anti-cancer agents (Lin *et al.* 2011a, Lee *et al.* 2016, Leith *et al.* 2017).

Resveratrol is a polyphenolic antioxidant enriched in grape skin and other medicinal plants, which exerts therapeutic effects against a wide range of diseases including cancer *in vitro* and *in vivo* (Tseng *et al.* 2004, Lin *et al.* 2011b, Varoni *et al.* 2016). Resveratrol binds to a receptor, situated on integrin $\alpha\beta_3$, to activate extracellular signal-regulated kinase-1 and -2 (ERK1/2) and to induce cyclooxygenase 2 (COX-2) nuclear accumulation, which is essential for resveratrol-induced anti-proliferation in several types of cancer cells (Chin *et al.* 2015). The nuclear activated complex of ERK1/2 and COX-2 stimulates Ser-15 phosphorylation of p53, which then induces apoptosis *via* the activation of its target genes in resveratrol-treated cells (Lin *et al.* 2008b, 2011b). Resveratrol-induced COX-2 nuclear accumulation can be inhibited by other hormone-like small molecules, which bind to integrin $\alpha\beta_3$, such as dihydrotestosterone (DHT) (Chin *et al.* 2015) and T_4 (Lin *et al.* 2007). Furthermore, resveratrol's anti-cancer properties are hindered by its low bioavailability, as it is rapidly and intensively metabolized by the intestine and the liver (Almeida *et al.* 2009, Cottart *et al.* 2010). Finding strategies to increase resveratrol's anti-proliferative efficacy against cancer, or in combination with other anti-cancer agents, is a potentially important path to investigate.

Against this background, we investigated whether tetrac potentiates resveratrol's anti-cancer properties in colorectal cancer; if it did so, we wanted to ascertain the molecular mechanisms involved. Results reported here indicate that tetrac-enhanced resveratrol-induced anti-proliferation in colon cancer by promoting nuclear COX-2 accumulation. The mechanisms implicated in this action involved the downregulation of nuclear β -catenin and HMGA2, which are capable of preventing

resveratrol-induced COX-2 nuclear accumulation. Concurrently, tetrac triggered chibby family member 1 nuclear accumulation, which may further compromise the nuclear β -catenin-dependent gene expression and proliferation.

Material and methods

Reagents

Tetrac and resveratrol were purchased from Sigma-Aldrich (Sigma-Aldrich). Resveratrol was prepared as a stock concentration of 100 nM in dimethyl sulfoxide (DMSO), and tetrac was dissolved in KOH-propylene glycol at a concentration of 10^{-2} M. Reagents were conserved at -20°C . The final concentrations of solvents used to dissolve the reagents were tested for activity and did not affect the results of the experiments.

Tissue samples and establishment of primary cultures of cancer cells

Human colon cancer samples were obtained from colon cancer patients admitted to Shuang-Ho hospital (Taipei, Taiwan). The enrolled patients did not receive any chemotherapy or radiation therapy prior to surgery. A written informed consent approved by the Institutional Review Board of Taipei Medical University and affiliated hospitals (IRB number N201603078) was signed by all patients. Collected tissue samples were maintained in serum- and penicillin (500 IU/mL)- and streptomycin (500 $\mu\text{g/mL}$)-containing saline at 4°C from the operating room to the research laboratory and processed immediately. Human colorectal primary cancer cells were established from the biopsies as previously described by Gharagozloo and coworkers (Gharagozloo *et al.* 2012).

Cell cultures

Human colorectal cancer HCT 116 (ATCC CCL-247) and HT-29 (ATCC HTB-38) cell lines were purchased from American Type Culture Collection (ATCC) by the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). These cell lines were tested and authenticated by the BCRC from which we acquired our cells. Primary colon cancer cells were established as described in the previous section, from colon cancer samples histologically confirmed by a medical pathologist. Primary cells were further analyzed for ki-67 expression and confirmed positive. HCT116 and HT-29 cells were

used from passage 5 to passage 15. Primary cells were employed immediately until passage 4. All cells were cultured in RPMI-1640 medium (Gibco, Invitrogen) that was supplemented with 10% FBS, penicillin 100 U/mL and streptomycin 100 $\mu\text{g/mL}$. All cells were maintained at 37°C in a humidified incubator with 5% CO_2 and 95% air.

Immunoblotting

To extract total proteins, cells were lysed in 1-fold radio-immunoprecipitation assay (RIPA) buffer-containing 1-fold protease inhibitor and 1-fold phosphatase inhibitor. Nuclear proteins were extracted following the Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Kit protocol (Thermo Scientific). The blotting technics are standard and have been described in several of our manuscripts (Chin *et al.* 2015, Lin *et al.* 2016a). Secondary antibodies were goat anti-rabbit IgG, rabbit anti-mouse IgG or rabbit anti-goat horseradish peroxidase, depending on the origin of the primary antibody. Immunoreactive proteins were detected using Amersham Imager 600 imaging system (GE Healthcare). Protein density was analyzed with ImageJ 1.5 software (NIH).

Real-time PCR

Total RNA was extracted from tumor tissues with Genezol (TriRNA Pure Kit (GZX050/GZX100/GZX200)|Geneaid) or from cultured cells with Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare Life Sciences). Genomic DNA was eliminated with DNase I. Two micrograms of total RNA were reverse-transcribed with RevertAid H Minus First Strand cDNA Synthesis Kit (Life Technologies) into cDNA and used as the template for real-time PCR reactions and analysis. The real-time PCR reactions were performed as described in our previous studies (Chin *et al.* 2015). The genes primers and accession numbers are provided in Table 1.

Cell proliferation assay

Cells (5×10^5 cells per well for HCT116 and HT-29, 5×10^3 cells for colon 260417) were seeded on 6-well trays and synchronized by 24-h starvation in serum-deprived RPMI-1640 medium. Cells were then treated or not for 72 h in 10% hormone-stripped FBS containing medium. Agents and medium were renewed daily. At the end of the experiments, cells were trypsinized and harvested for count with Countess automated cell (Invitrogen, Thermo Fisher Scientific).

Table 1 Genes primers sequences and accession numbers.

Gene	Accession number	Forward primer (5'→3')	Reverse primer (5'→3')
<i>CTNNB1</i>	NM_001904	CTGGTCCTTTTGGTCGAGGA	GCAAGGCTAGGGTTTGATAAAT
<i>HMGA2</i>	NM_003483.4	GTTGGTCGCAGCTACATAAGA	CAAAGGAACAGGGAGAAAGTCA
<i>HIF1A</i>	NM_001243084.1	TGAACGTCGAAAAGAAAAGTCTCG	GGAAGTGGCAACTGATGAGC
<i>MCL1</i>	NM_021960.4	CCAAGAAAGTCGATCGAACC	CAAACCCATCCCAGCCTCTT
<i>XIAP</i>	NM_001167.3	CTGGCGCGAAAAGGTGGA	TGATGTCTGCAGGTACACAAGT
<i>CCND1</i>	NM_053056	CAAGGCCTGAACCTGAGGAG	GATCACTCTGGAGAGGAAGCG
<i>c-Myc</i>	NM_002467	TTCGGGTAGTGGAAAACAG	CAGCAGCTCGAATTCTTCC
<i>PCNA</i>	BC062439.1	TCTGAGGGCTTCGACACCTA	TCATTGCCGGCGCATTTTAG
<i>MMP2</i>	NM_004530.5	ATCCAGACTTCCTCAGGCGG	CCTGGCAATCCCTTTGTATGTT
<i>MMP9</i>	NM_004994.2	TGTACCCTATGGTTACACTCG	GGCAGGGACAGTTGCTTCT
<i>MMP13</i>	NM_002427.3	CCAGACTTCACGATGGCATTG	GGCATCTCCTCCATAATTTGGC
<i>VEGFA</i>	NM_001204384.1	TACCTCCACCATGCCAAGTG	GATGATTCTGCCCTCTCCTT
<i>FGF2</i>	NM_002006.4	GAGAAGAGCGACCTCACA	TAGCTTTCTGCCAGGTCC
<i>ITGAV</i>	NM_002210.4	TCCGATTCCAACTGGGAGC	AAGGCCACTGAAGATGGAGC
<i>ITGB3</i>	NM_000212.2	CTGGTGTTCACACTGATGCCAAG	TGTTGAGGCAGGTGGCATTGAAGG
<i>R18S</i>	M10098	GTAACCGTTGAACCCATT	CCATCCAATCGGTAGTAGCG

MTT assay (cell viability assay)

Colon cancer cells (1×10^3 cells per well) were seeded in 96-well plates and synchronized by 24-h starvation in serum-deprived RPMI-1640 medium. Cells were then treated or not for 72 h in 10% hormone-stripped FBS-containing medium. Agents and medium were renewed on a daily basis. Cell proliferation was determined by incubating the cells with 200 μ L of fresh medium containing 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) for 4 h at 37°C. After removal of the MTT solution, the resulting formazan crystals were dissolved completely in dimethyl sulfoxide (DMSO) and the plates were read using a microplate reader (Anthos 2010; Biochrom, Cambridge, United Kingdom) by measuring the absorbance at 540 nm. Quintuplicate wells were assayed for each experiment, and three independent experiments were performed.

Small interfering RNA transfection

Colon cancer cells were seeded in 6-well tissue culture plates (10^5 cells/well), grown at 60%~80% confluence and maintained in an antibiotic-free medium for 24 h before transfection. Just prior to transfection, the culture medium was removed, and cells were washed once with PBS, then transfected with HMGA2 siRNA (Thermo Fisher) or β -catenin siRNA (Cell Signaling Technology) with Lipofectamine 3000, in Opti-MEM I medium according to the manufacturer's instructions. After transfection, cultures were incubated at 37°C for 4 h and then placed in fresh culture medium for an additional 48 h. Transfected cells were then treated with agents for Western blots experiments (24 h) or cell proliferation assays (72 h).

Immunohistochemistry

Tissue sections were dewaxed in xylene (Sigma-Aldrich) and rehydrated in alcohol (Sigma-Aldrich). Antigen retrieval was carried out by incubating tissue sections in citrate antigen retrieval buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0) at 95°C for 40 min in a water bath. Endogenous peroxidase was blocked by incubation with Peroxidase Block and Protein Block (Novocastra Laboratories, Wetzlar, Germany) at room temperature, both for 5 min. After 3 washes with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBST), slides were individually incubated for overnight with COX-2 (Santa Cruz 1745 1:200 dilution), HMGA2 (Genetex GTX100519; 1:200 dilution), β -catenin (BD Bioscience 1:200 dilution) or chibby family member 1 ((s-13) Sc-86379 1:200 dilution) antibodies at 4°C. Tissue sections were then rinsed in PBST and post primary block (Novocastra Laboratories) was applied at room temperature for 15 min, followed by a NovoLink Polymer Detection System (Novocastra Laboratories) for another 15 min at room temperature. Subsequently, slides were treated with 3,3'-diaminobenzidine chromogen (Novocastra Laboratories) for 5 min and counterstained with hematoxylin (Novocastra Laboratories), then mounted and examined under Nikon Eclipse ci optical microscope imaging system (Nikon Eclipse ci, Nikon Instruments). Images were captured at magnification 400 $\times$ with a scale bar of 50 μ m.

Confocal microscopy

Exponentially growing primary colon cancer 260417 cells were seeded on sterilized cover glasses (Paul Marienfeld, Lauda-Königshofen, Germany). After 24 h of starvation,

cells were treated with tetrac 10^{-7} M, resveratrol $10\mu\text{M}$ or their combination for another 24h. Cells were fixed with 4% paraformaldehyde in PBS for 10min and then permeabilized in 0.06% Triton X-100 for 5 min. The cells were incubated with a monoclonal antibody to β -catenin (ab16051; 1:500) or HMGA2 (Genetex; 1:400) followed by an incubation with Alexa-488-labeled goat anti-rabbit (ab150077; 1:500). Cells were next incubated in Hoechst stain (Hoechst 33258 Sigma-Aldrich 1:1000) and mounted in EverBrite Hardset mounting medium. The fluorescent signals from β -catenin and HMGA2 were recorded and analyzed with the TCS SP5 Confocal Spectral Microscope Imaging System (Leica Microsystems).

Nude mouse ectopic xenograft model

Female nude homozygous mice (BALB/cAnN.Cg-Foxn1nu/CrlNarl) aged 5–6 weeks, with an average body weight of 22kg, were purchased from the National Laboratory of Animal Center (Taipei, Taiwan). All mice were handled in accordance with protocols approved by the Institutional Animal Care and Use Committee, National Defense Medical Center, Taipei, Taiwan (IACUC-15-340). Mice were inoculated subcutaneously in each flank, with 2×10^7 human colon cancer HCT 116 cells. At the onset of the tumors, animals were randomly assigned to 6 treatment groups containing 5 mice each: control; resveratrol 2.5mg/kg per body weight; resveratrol 25mg/kg; tetrac 1mg/kg; tetrac 1mg/kg combined with resveratrol 2.5mg/kg and tetrac 1mg/kg combined with resveratrol 25mg/kg. Tetrac and resveratrol were administered intraperitoneally, five days a week for resveratrol and twice a week for tetrac. Tumor volume and animal weight were measured once a week, and the tolerance of agents was evaluated on a daily basis. Animals were killed on day 21, as the tumor burden in the control group was unbearable for animals, in compliance with institutional guidelines. Half of the tumor tissue was formalin-fixed and paraffin-embedded for immunohistochemistry. The other half was snap frozen in liquid nitrogen and stored at -80°C for subsequent experiments.

Statistical analysis

Data were analyzed with IBM SPSS Statistics software, version 20.0 (SPSS). Statistical significance between multiple experimental groups was analyzed by one-way

ANOVA followed by a Bonferroni correction. P value < 0.05 was considered as statistically significant.

Results

Tetrac potentiates resveratrol-induced anti-proliferation and COX-2 nuclear accumulation in colon cancer cells

Both tetrac and resveratrol inhibit cancer cell proliferation (Lin *et al.* 2011a). In order to assess the anti-proliferation induced by tetrac, resveratrol and their combination in colorectal cancer, HCT116 and HT-29 cancer cells were treated with different concentrations of resveratrol (1, 10 or $100\mu\text{M}$), tetrac 10^{-7} M alone or combined to resveratrol ($1\mu\text{M}$ or $10\mu\text{M}$) for 72h, after 24h of starvation. Tetrac at a concentration of 10^{-7} M significantly inhibited colorectal cancer cell proliferation (Fig. 1A and B). Similarly, resveratrol inhibited cancer cell proliferation in a concentration-dependent manner. Interestingly, tetrac enhanced the anti-proliferation effect of resveratrol in the combination treatments. Parallel studies on primary colon cancer 260417 cell cultures showed similar results (Fig. 1C). These results were further confirmed by MTT assays (Supplementary Fig. 1A, B and C, see section on supplementary data given at the end of this article).

Binding to its receptor situated on integrin $\alpha\beta_3$, resveratrol induces apoptosis in cancer cells via activation of the ERK1/2 pathway. This leads to ERK1/2 phosphorylation and nuclear translocation, as well as nuclear accumulation of inducible COX-2, which is essential for integrin-dependent resveratrol-induced apoptosis (Lin *et al.* 2011b). To assess whether tetrac promotes resveratrol-induced COX-2 nuclear accumulation, colorectal cancer HCT116 (Fig. 1D) and HT-29 (Fig. 1E) cells were treated with resveratrol ($1\mu\text{M}$ or $10\mu\text{M}$), tetrac 10^{-7} M or their combination for 24h and nuclear proteins were extracted for Western blot analysis. Resveratrol, but not tetrac, induced COX-2 nuclear accumulation and p53-Ser15 phosphorylation (Fig. 1D and E). Noticeably, resveratrol-induced nuclear COX-2 accumulation and p53-Ser15 phosphorylation were enhanced with tetrac co-treatment (Fig. 1D and E). Parallel studies conducted in human primary colon cancer cell 260417 cultures showed comparable results (Fig. 1F). In addition, all cells expressed integrin $\alpha\beta_3$ subunits (Supplementary Fig. 2).

Figure 1

Effects of tetrac and resveratrol on colon cancer cells proliferation and COX-2 nuclear accumulation. Human colorectal cancer cell lines HCT116 (A), HT-29 (B) and a primary colon cancer cell, colon 260417 (C) were treated with different concentrations of resveratrol (1, 10 or 100 μ M), tetrac 10^{-7} M alone or combined with resveratrol (1 μ M or 10 μ M) for 72 h and the total cell number was counted using a cell counter. Cells were maintained in serum-deprived medium for 24 h prior to agents' treatment. Data are displayed as the mean $\pm$ s.d. * P <0.05, ** P <0.01, *** P <0.001 compared with control, # P <0.05, ### P <0.001 compared with tetrac 10^{-7} M alone, \$ P <0.01, \$\$\$ P <0.001 compared with resveratrol alone at the same concentration. Human colorectal cancer cell lines HCT116 (D), HT-29 (E) and a primary colon cancer cell 260417 (F) were treated with different concentrations of resveratrol (1 μ M and 10 μ M), tetrac 10^{-7} M alone or in combination with resveratrol for 24 h, after 24 h of starvation. Nuclear proteins were extracted and Western blot experiments were performed. Protein density was analyzed with ImageJ and normalized to lamin B1. Resveratrol-induced COX-2 nuclear accumulation was enhanced by tetrac co-treatment. Representative results of three experiments are displayed. Data are displayed as the mean $\pm$ s.d. * P <0.05, ** P <0.01, *** P <0.001 compared with control, # P <0.01, ### P <0.001 compared with resveratrol 1 μ M alone, \$ P <0.05, \$\$\$ P <0.001 compared with resveratrol 10 μ M alone.

Tetrac inhibits actions of HMGA2 and β -catenin, but promotes chibby family member 1 nuclear accumulation in colon cancer cells

The oncogenic potential of HMGA2 has been demonstrated in almost all steps of carcinogenesis (Fusco & Fedele 2007). T_4 has been shown to induce the expression of HMGA2 in colorectal cancer HCT116 cells and to trigger nuclear translocation of HMGA2 and β -catenin in both HT-29 and HCT116 cells (Lin 2016). Given the antagonistic effect of tetrac on T_4 -induced cancer cell proliferation, we hypothesized that tetrac might affect the amount of HMGA2 in the nucleus. To verify this hypothesis, colon cancer HCT116 (Fig. 2A), HT-29 (Fig. 2B) cell lines and a primary colon cancer cell 310816 (Fig. 2C) were treated with different concentrations of tetrac for 24 h, and nuclear proteins were extracted for Western blot analysis. As expected, tetrac inhibited HMGA2 nuclear protein amount in a concentration-dependent manner in all cell

lines. Tetrac also downregulated PI3K phosphorylation (Fig. 2A, B and C), which has been identified as one of the key activators of HMGA2 expression in cancer settings (Ayoubi *et al.* 1999, Zentner *et al.* 2001).

Wnt/ β -catenin signaling is frequently dysregulated in colon cancer (Basu *et al.* 2016). Tetrac has been previously shown to induce *CBY1* gene expression in breast cancer cells (Glinskii *et al.* 2009). Tetrac also antagonizes T_4 , which has been proven to induce β -catenin nuclear accumulation. Against this background, we sought to determine the effect of tetrac on the nuclear content of both β -catenin and its nuclear antagonist, chibby family member 1. To this end, colon cancer HCT116 and HT-29 cells were treated with different concentrations of tetrac for 24 h and nuclear proteins were extracted for Western blot analyses. Tetrac reduced nuclear content of β -catenin, but increased chibby family member 1 accumulation in both cells (Fig. 2A and B). Parallel studies conducted in

Figure 2

Tetrac regulates the nuclear accumulation of β -catenin, HMGA 2 and chibby family member 1 and the phosphorylation of PI3K. Human colorectal cancer cell lines, HCT116 (A), HT-29 (B) and a primary colon cancer cell culture 310816 (C) were treated with different concentration of tetrac (10^{-8} M to 10^{-6} M) for 24 h, after 24 h of starvation. Western blot analyses were performed using nuclear proteins for β -catenin, HMGA 2 and chibby family member 1 and whole-cell lysates for pPI3K. Nuclear proteins were normalized to lamin B1 and whole-cell proteins to β -actin. Tetrac decreased the nuclear accumulation of β -catenin and HMGA 2 and the phosphorylation of PI3K, but enhanced the nuclear amount of chibby family member 1. Representative results of three experiments are displayed. Data are displayed as the mean $\pm$ s.d., at the level of significance *** P < 0.001 compared with control, ### P < 0.001 compared with tetrac 10^{-8} M, \$\$\$ P < 0.01, \$\$\$\$ P < 0.001 compared with compared with tetrac 10^{-7} M.

primary colon cancer cells 310816 showed comparable results (Fig. 2C).

To further explore the effect of tetrac on β -catenin and HMGA2 in colon cancer cells, confocal microscopy experiments were conducted. Primary cultures of human colon cancer cells 260417 were treated with tetrac 10^{-7} M, resveratrol 10 μ M or their combination for 24 h. This primary culture of human colon cancer cells exhibited high concentrations of both β -catenin and HMGA2, which was significantly reduced upon exposure to tetrac, alone or in combination with resveratrol (Fig. 3A and B).

Silencing of HMGA2 or β -catenin promotes resveratrol-induced COX-2 nuclear accumulation and anti-proliferation

We have shown that tetrac potentiates resveratrol-induced COX-2 nuclear accumulation, p53-Ser15 phosphorylation and subsequent anti-proliferation. We also proved that tetrac not only downregulated oncogenic HMGA2 and β -catenin but also potentiated the nuclear accumulation of chibby family member 1. We then hypothesized that tetrac potentiation of resveratrol-induced COX-2 nuclear accumulation might depend upon HMGA2 and β -catenin downregulation. To examine these possibilities, HCT116

and HT-29 cells were treated with β -catenin siRNA or HMGA2 siRNA for 48 h and then treated or not with agents for an additional 24 h for Western blot analyses or 72 h for cell count assays. Nuclear proteins were extracted for Western blot analysis. Interestingly, the nuclear COX-2 accumulation and subsequent anti-proliferation induced by resveratrol was enhanced by both HMGA2 and β -catenin knockdown (Fig. 4A, B, C and D). These results indeed suggested that tetrac potentiated resveratrol-induced COX-2 nuclear accumulation by reducing the nuclear abundance of HMGA2 and β -catenin.

Tetrac potentiates resveratrol anti-cancer activities in a nude mice xenograft model

To investigate the potentiating effect of tetrac on resveratrol's anti-cancer effect *in vivo*, we conducted a mouse subcutaneous model of colon cancer. Both tetrac and resveratrol induced a significant anti-tumoral effect (Fig. 5A, B, D and Table 2). Resveratrol inhibited tumor growth in a concentration-dependent manner (52.57% for resveratrol 2.5 mg/kg vs 80.99% for resveratrol 25 mg/kg) (Table 2). At 1 mg/kg, tetrac showed an inhibitory effect comparable to that of resveratrol at 25 mg/kg (78.93% vs 80.99%) (Table 2). Although not

Figure 3

Tetrac reduces the accumulation of β -catenin and nuclear HMGA2. Human primary colon cancer cell cultures 260417 were treated with tetrac 10^{-7} M, resveratrol $10 \mu\text{M}$ or their combination for 24h and cells were examined by confocal microscopy. Tetrac downregulated total β -catenin alone or combined to resveratrol, as shown by the reduction of the green fluorescence (A). Furthermore, tetrac downregulated the nuclear protein HMGA2 (B) as indicated by the appearance of yellow fluorescence due to the superimposition of HMGA2 (green fluorescence) and nucleus (red fluorescence).

Figure 4

Silencing β -catenin or HMGA2 promotes resveratrol-induced COX-2 nuclear accumulation and antiproliferation. Human colorectal cancer cell lines, HCT116 (A and B) and HT-29 (C and D) were treated or not, with either siRNA of β -catenin or siRNA of HMGA2 for 48h, then were treated with agent for 24h for Western blot analysis or 72h for cell count assay. Nuclear proteins were extracted and Western blot analyses were performed. Nuclear COX-2 was normalized to lamin B1. Resveratrol-induced COX-2 nuclear accumulation and anti-proliferation was enhanced by both β -catenin and HMGA2 silencing. Representative results of three experiments are shown. Data are displayed as the mean $\pm$ s.d. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control (without siRNA treatment), # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared with siRNA control alone, \$\$\$ $P < 0.01$, \$\$\$\$ $P < 0.001$ compared with resveratrol $10 \mu\text{M}$ alone (without siRNA).

Figure 5

Tetrac and resveratrol inhibit cancer growth in mouse xenograft model. Human colon cancer HCT 116 cells (2×10^7) were injected bilaterally into nude mice. The tumor volume was normalized to the volume at treatment start. (A) Growth curve of tumors over time. (B) Between group comparison of final tumor volumes. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with control, $\#P < 0.05$, compared with tetrac alone, $^{ss}P < 0.01$, compared with resveratrol 25 mg/kg alone. (C) Body weight. (D) Representative individuals of each group are shown.

statistically significant ($P = 0.244$), the combination of tetrac and resveratrol 2.5 mg/kg displayed a greater inhibitory effect compared to single agents at the same concentration (85.77% vs 78.93% and 52.57% for tetrac 1 mg/kg and resveratrol 2.5 mg/kg, respectively)

Table 2 Tumor growth inhibition percentages.

Treatment groups	Final tumor volume (mm ³)	Inhibition (%) [*]
Control	1812.5968 $\pm$ 379.95	–
Tetrac 1 mg/kg	381.9487 $\pm$ 116.62	78.92809
Resveratrol 2.5 mg/kg	859.5766 $\pm$ 237.51	52.57762
Resveratrol 25 mg/kg	344.5496 $\pm$ 75.78	80.99138
Tetrac 1 mg/kg + Resveratrol 2.5 mg/kg	258.0172 $\pm$ 149.52	85.76533
Tetrac 1 mg/kg + Resveratrol 25 mg/kg	52.4302 $\pm$ 30.82	97.10745

^{*}Percentage reduction of tumor volume in the treatment groups as compared to the control group. Tumor volumes are presented as the mean $\pm$ S.D.

(Table 2). The combination of high concentrations of resveratrol (25 mg/kg) and tetrac showed a potentiating effect, which was statistically significant compared to the anti-tumoral effect of single agents ($P = 0.012$ vs tetrac, $P = 0.006$ vs resveratrol 25 mg/kg). More importantly, this combination inflected the tumor growth curve from the initial volume as displayed in the growth curve over time (Fig. 5A). All agents were well tolerated and did not affect the body weight of xenografted animals (Fig. 5C).

To further explore the molecular mechanisms implicated in tetrac's potentiation of resveratrol anti-cancer effect *in vivo*, we performed immunohistochemical staining of the xenograft tumor samples. Resveratrol-induced COX-2 nuclear accumulation, which was further enhanced by tetrac co-treatment (Fig. 6A). Likewise, tetrac reduced β -catenin and HMGA2 nuclear amount but enhanced chibby family member 1 nuclear accumulation (Fig. 6B).

Figure 6

Tetrac and resveratrol regulate the accumulation of COX-2, β -catenin, HMGA2 and chibby family member 1 in tumor tissues. (A) Nuclear COX-2 expression (purple dots) was detected using IHC staining. (B) The expression of nuclear β -catenin (purple dots), nuclear HMGA2 (brown dots), and nuclear chibby family member 1 (purple dots) on tumor cells were also detected. Magnification 400x, scale bar 50 μ m.

Taken together, these results indeed suggested that tetrac's potentiating effect on resveratrol-induced anti-proliferation is subservient, on the one hand to the downregulation of nuclear β -catenin and nuclear HMGA2, and on the other hand to the increase of chibby family member 1 nuclear amount (Fig. 7).

Tetrac and resveratrol affect the expression of cancer cell-regulated genes *in vivo*

Next, we sought to further ascertain the synergistic effect of agents at the molecular level *in vivo*, by means of qPCR analyses using the harvested tumor samples. Both tetrac and resveratrol inhibited (a) *CTNNB1*, *HMGA2* and *HIF1A* gene expression (Fig. 8A), (b) the apoptosis-related genes *MCL1* and *XIAP* (Fig. 8B), (c) the proliferative genes *CCND1*, *c-Myc* and *PCNA* (Fig. 8C) (d) the metastasis-support genes *MMP2*, *MMP9* and *MMP13* (Fig. 8D) and the angiogenic genes *VEGFA* and *FGF2* (Fig. 8E). A synergistic effect was also observed on most of these genes. These results indicated that, beyond its potentiating effect on resveratrol-induced COX-2 nuclear accumulation, tetrac expresses highly complex anti-tumoral effects *in vivo*.

Discussion

In this study, we have demonstrated that tetrac potentiates resveratrol-induced anti-proliferation in colorectal cancer cells. This potentiating effect was mediated, at least in part, by the inhibition of the nuclear accumulation of HMGA2 and β -catenin, and the increase of chibby family member 1. Both tetrac and resveratrol have been proven to induce anti-proliferation in different cancer cell lines. The current study is the first to assess the synergistic effect of resveratrol and tetrac in combination therapy in colon cancer, highlighting the implication of HMGA2, β -catenin and its nuclear antagonist chibby family member 1. Knockdown of either HMGA2 or β -catenin enhanced resveratrol-induced COX-2 nuclear accumulation, which has been shown to be essential for integrin $\alpha\beta 3$ -mediated resveratrol-induced apoptosis in cancer cells (Lin *et al.* 2011b). Elsewhere, tetrac has been demonstrated to sensitize cancer cells to other anti-cancer agents or radiations (Rebbaa *et al.* 2008, Lee *et al.* 2016, Leith *et al.* 2017), underlining the potential of this anti-cancer agent in therapeutic combination.

There is a complex interplay between HMGA2 and Wnt signaling, both in physiological settings and in cancer.

Figure 7

Molecular mechanisms. Resveratrol induces ERK1/2 phosphorylation and nuclear translocation, as well as COX-2 nuclear accumulation. Phosphorylated ERK1/2 cooperates with nuclear COX-2 to induce p53 phosphorylation at Serine 13 and subsequent anti-proliferation in cancer cells. Tetrac on the one hand, downregulates nuclear β -catenin and nuclear HMGA2, and on the other hand increases chibby family member 1 nuclear amount, which will further compromise β -catenin actions at its target genes promoters. Nuclear β -catenin and nuclear HMGA2 antagonize resveratrol-induced COX-2 nuclear accumulation, which is essential for resveratrol-induced apoptosis in cancer cells.

HMGA2 triggers senescence in human normal lung fibroblast WI-38 cells through downregulation of Wnt signaling (Shi *et al.* 2017). In malignancies, HMGA2 drives epithelial-to-mesenchymal transition (EMT) in gastric cancers via activation of Wnt/ β -catenin pathway (Zha *et al.* 2013). Furthermore, HMGA2 silencing impairs breast tumor development in Wnt1 transgenic mice (Morishita *et al.* 2013). However, other studies also indicate that WNT10B/ β -catenin pathway controls HMGA2 expression in triple-negative breast cancer cells (Wend *et al.* 2013). Therefore, there is a mutually promoting relationship between HMGA2 and Wnt to stimulate cancer progression. In our study, we found that tetrac inhibited the nuclear accumulation of both HMGA2 and β -catenin both *in vitro* and in *in vivo* models.

The relationship between thyroid dysfunction and cancer has long been an important topic in clinical investigations (Liao *et al.* 2010, Moeller & Führer 2013).

Although some discrepancies in results have been found (Hercbergs *et al.* 2010), partially owing to differences in experimental designs, epidemiological studies involving large cohorts suggest a causal link between hyperthyroidism and cancer (Khan *et al.* 2016). The biological plausibility of these epidemiological observations is based on the fact that T_4 exerts non-genomic activities that promote cancer cell proliferation via its receptor on integrin $\alpha\beta 3$. At the same site, T_4 is able to impair resveratrol's anti-cancer features (Lin *et al.* 2008a). However, tetrac, which shares a structural analogy with T_4 , can compete at the same cell surface receptor and antagonize in the presence of T_4 (Lin *et al.* 2008a). The identification of this new mechanism involving HMGA2, β -catenin and chibby family member 1 therefore suggests that the potentiation of the effect of resveratrol by tetrac may be much more important in situations of thyroid dysfunction with high T_4 production.

Similar to resveratrol, tetrac is a multi-target anti-cancer agent. Indeed, tetrac modulates the expression of several genes in cancer cells, which govern cell survival, angiogenesis and metastasis (Davis *et al.* 2014). In the present study, we found that tetrac and resveratrol downregulated *CTNNB1*, *HMGA2* and *HIF1A* genes expression (Fig. 8A). Both agents also downregulated the anti-apoptotic genes *XIAP*, *MCL1*, the proliferative genes *c-Myc*, *CCND1* and *PCNA*, the angiogenic genes *VEGFA* and *FGF2* and the metastatic genes *MMP2*, *MMP9* and *MMP13* (Fig. 8B, C, D and E), which is consistent with the findings of previous studies (Glinskii *et al.* 2009, Davis *et al.* 2014). Furthermore, the combination of resveratrol and tetrac showed a synergistic effect on most of the above-mentioned genes, suggesting that the mechanisms involved in the living organism are far more complex and outweigh the potentiation of nuclear COX-2 accumulation alone.

Since the seminal work of Jang and coworkers (Jang *et al.* 1997), a wealth of knowledge has been collected on the anti-cancer properties of resveratrol *in vitro* and *in vivo*. However, pharmacokinetics studies soon revealed a potential drawback for the translation of the stilbene into clinical use. Indeed, extensive intestinal and hepatic metabolisms undermine the systemic bioavailability of unchanged resveratrol, which is otherwise, satisfactorily absorbed (Walle *et al.* 2004). Therefore, it has been speculated that if resveratrol directly acts on the intestinal epithelium, the anti-cancer potential of the stilbene in distant organs might be attributable to its sulfate and glucuronide metabolites, which retain some biological activities (Hoshino *et al.* 2010). Elsewhere, resveratrol is well tolerated in healthy individuals, but some serious

Figure 8

Tetrac and resveratrol regulate the expression of cancer genes *in vivo*. The level of expression of genes was detected by qPCR experiments. (A) *CTNNB1*, *HMGA2*, *HIF1A*. (B), Apoptosis-related genes *MCL1*, *XIAP*. (C) Proliferation-related genes *CCND1*, *c-Myc*, *PCNA*. (D) Metastatic genes *MMP2*, *MMP9*, *MMP13*. (E) Angiogenesis-related genes *VEGFA* and *FGF2*. Data are shown as the mean $\pm$ s.d. of three experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control, # $P < 0.05$, ### $P < 0.001$ compared with tetrac 1 mg/kg alone, \$ $P < 0.05$, \$\$\$ $P < 0.001$ compared with resveratrol 25 mg/kg alone.

adverse effects have been noted in already-diseased people (Boocock *et al.* 2007, Patel *et al.* 2011, Popat *et al.* 2013, Tani *et al.* 2014). In animal studies, several concentrations have been described in the literature, as summarized elsewhere (Baur & Sinclair 2006, Carter *et al.* 2014). In our study, we wanted to compare the potentiating effect of tetrac on resveratrol's anti-cancer abilities, at low dose 2.5 mg/kg (similar to Kimura & Okuda 2001), and at a higher dose, 25 mg/kg (similar to Garvin *et al.* 2006). The intraperitoneal injection was done to prevent the massive biodegradation undergone by resveratrol in the small intestine when orally administered, but also to hold control of the concentrations effectively administered to animals. Tetrac, which is a naturally occurring derivative of T_4 , represents less than 1% of circulating thyroid hormones (Davis *et al.* 2013). In physiological conditions, tetrac exerts a thyro-mimetic effect and inhibits the

release of thyroid-stimulating hormone (TSH) by the pituitary gland (Burger *et al.* 1979, Moreno *et al.* 2008). Clinical trials with tetrac at therapeutic doses are yet to be conducted to assess the full spectrum of its biological effect on human being. In animal models, tetrac and its nanoparticulate analog nano-diamino-tetrac did not induce adverse effects (Yalcin *et al.* 2009). In our study, tetrac 1 mg/kg was used in accordance with previous studies (Yalcin *et al.* 2009, Mousa *et al.* 2012, Yoshida *et al.* 2012). Both resveratrol and tetrac were well tolerated, and no adverse effect was observed (Fig. 5C).

In summary, this study aimed at deciphering whether tetrac, the de-aminated analog and antagonist of T_4 , potentiates the anti-cancer ability induced by resveratrol. We found that tetrac promoted resveratrol-induced anti-proliferation in colon cancer cell lines, in colon cancer primary cells and enhanced the anti-tumoral effect of

resveratrol in a mouse xenograft model of colon cancer. Western blot and immunohistochemistry analyses showed that tetrac enhanced resveratrol-induced nuclear COX-2 accumulation, which has been described as essential for resveratrol-induced apoptosis in cancer cells. Tetrac inhibited the nuclear accumulation of both HMGA2 and β -catenin, but enhanced chibby family member 1, the nuclear β -catenin antagonist. Silencing either β -catenin or HMGA2 promoted resveratrol-induced COX-2 nuclear accumulation and anti-proliferation, suggesting that tetrac potentiated resveratrol's anti-cancer features via inhibition of HMGA2 and β -catenin nuclear accumulation. Additionally, tetrac and resveratrol individually and synergistically inhibited several genes relevant to cancer growth, angiogenesis and metastasis *in vivo*.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-17-0450>.

Declaration of interest

Co-authors Paul J Davis and Shaker A Mousa are co-inventors of Nanotetrac, a nanoparticulate analog of tetrac. The other authors declare no conflict of interest to disclose.

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Author contribution statement

Concept and design of experiments: A W N and H Y L. Acquisition of data: A W N, Y T C, C Y L, Y J S, and Y R C. Analysis and interpretation of data: A W N, Y T C, C Y L, J Z P, S I, and H Y L. Writing and review of the manuscript: A W N, Y T C and H Y L. Supervision of the work: H Y L, W S L, E F, S A M, C A C, L F L, J A B, Y H, J W P and P J D.

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