Natural polyphenols facilitate elimination of HT-29 colorectal cancer xenografts by chemoradiotherapy: a Bcl-2- and superoxide dismutase 2-dependent mechanism

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Abstract
Colorectal cancer is one of the most common malignancies worldwide. The treatment of advanced colorectal cancer with chemotherapy and radiation has two major problems: development of tumor resistance to therapy and nonspecific toxicity towards normal tissues. Different plant-derived polyphenols show anticancer properties and are pharmacologically safe. In vitro growth of human HT-29 colorectal cancer cells is inhibited (~56%) by bioavailable concentrations of trans-pterostilbene (trans-3,5-dimethoxy-4'-hydroxystilbene; t-PTER) and quercetin (3,3',4',5,6-pentahydroxyflavone; QUER), two structurally related and naturally occurring small polyphenols. i.v. administration of t-PTER and QUER (20 mg/kg/day) inhibits growth of HT-29 xenografts (~51%). Combined administration of t-PTER + QUER (20 mg/kg/day) inhibits growth of HT-29 xenografts (~51%). Combined administration of t-PTER + QUER, FOLFOX6 (oxaliplatin, leucovorin, and 5-fluorouracil; a first-line chemotherapy regimen), and radiotherapy (X-rays) eliminates HT-29 cells growing in vivo leading to long-term survival (>120 days). Gene expression analysis of a Bcl-2 family of genes and antioxidant enzymes revealed that t-PTER + QUER treatment preferentially promotes, in HT-29 cells growing in vivo, (a) superoxide dismutase 2 overexpression (~5.7-fold, via specificity protein 1-dependent transcription regulation) and (b) down-regulation of bcl-2 expression (~3.3-fold, via inhibition of nuclear factor-κB activation). Antisense oligodeoxynucleotides to human superoxide dismutase 2 and/or ectopic bcl-2 overexpression avoided polyphenols and chemoradiotherapy-induced colorectal cancer elimination and showed that the manganese-type superoxide dismutase and Bcl-2 are key targets in the molecular mechanism activated by the combined application of t-PTER and QUER. [Mol Cancer Ther 2008;7(10):3330–42]

Introduction
Colorectal cancer is the third most common cancer and the fourth most frequent cause of cancer deaths worldwide (1). Treatment of patients with recurrent or advanced colorectal cancer depends on the location of the disease. For patients with locally recurrent and/or liver-only and/or lung-only metastatic disease, surgical resection, if feasible, is the only potentially curative treatment, whereas patients with unresectable disease are treated with systemic chemotherapy.3 Currently, several first-line and second-line chemotherapy regimens are available that can be used in patients with recurrent or advanced colorectal cancer. The newer colorectal cancer chemotherapy schemas are serving as the platform on which combined novel targeted agents are based. Accepted first-line regimens are either irinotecan-based (IFL, FOLFIRI, and AIO) or oxaliplatin-based (FOLFOX4 and FOLFOX6).3 Combined chemotherapy and radiation therapy is used in rectal cancer-bearing patients, although improvements in the outcome of colon cancer-bearing patients treated with radiation therapy have not been proved. Survival for patients with advanced colorectal cancer is ~2 years on average, and there is an ongoing need for the identification of new therapeutic agents and/or treatment strategies (2).

Different polyphenolic compounds of natural origin, such as trans-resveratrol (trans-3,5,4'-trihydroxystilbene; t-RESV), have been studied for their potential antitumor properties (3). Cancer chemopreventive activity of t-RESV was first reported by Jang et al. (4). However, antitumor properties of t-RESV are limited due to its low systemic bioavailability (5). Thus, structural modifications of the t-RESV molecule appeared necessary to increase the bioavailability while preserving its biological activity.

Recently, we observed that trans-pterostilbene (trans-3,5-dimethoxy-4'-hydroxystilbene; t-PTER) and quercetin (3,3',4',5,6-pentahydroxyflavone; QUER) showed in vivo longer half-life than t-RESV and that their combination strongly inhibited metastatic growth of the highly malignant murine B16 melanoma F10 (B16M-F10; ref. 6). t-PTER...
and QUER inhibit bcl-2 expression in B16M-F10 cells, which sensitizes them to vascular endothelium-induced cytotoxicity (6). Indeed, at the molecular level, natural polyphenols have been reported to modulate several key elements in cellular signal transduction pathways linked to the apoptotic process (caspases and bcl-2 genes; ref. 7). Moreover, recent reports showed that polyphenolic compounds from blueberries, tea, or red wine can inhibit human colon cancer cell proliferation and induce apoptosis in vitro (8–10). Nevertheless, whether natural polyphenols may have useful applications in oncotherapy, and in colorectal cancer therapy in particular, remains to be investigated. For the present report, our aim was to study if t-PTER and/or QUER, by overcoming antiapoptotic responses, may potentiate the effect of chemotherapy and/or ionizing radiations on human HT-29 colorectal cancer cells growing in vivo.

Materials and Methods

Cell Culture

HT-29 human colon cancer cell lines were obtained from the American Type Culture Collection. HT-29 cells were grown in DMEM (Invitrogen; pH 7.4) supplemented with 10% FCS (Biochrom KG), 100 units/mL penicillin, and 100 μg/mL streptomycin. Cultures were maintained at 37°C in a humidified atmosphere with 5% CO₂. Cells were harvested by incubation for 5 min with 0.05% (w/v) trypsin (Sigma) in PBS [10 mmol/L sodium phosphate, 4 mmol/L KCl, and 137 mmol/L NaCl (pH 7.4)] containing 0.3 mmol/L EDTA followed by the addition of 10% FCS to inactivate the trypsin. Cell numbers were determined using a Coulter counter (Coulter Electronic). Cellular viability was assessed, as reported previously (5), by measuring trypan blue exclusion and leakage of lactate dehydrogenase activity.

Polyphenols

t-PTER was synthesized in our laboratory following standard Wittig and Heck reactions,4 whereas QUER was obtained from the Sigma.

Assessment of Cell Cycle Distribution

Analysis was done using a MoFlo High-Performance Cell Sorter (DAKO). Fluorochrome excitation was done with an argon laser tuned at 488 nm. Forward-angle and right-angle light scattering were measured. Data were acquired for 10⁴ individual cells. Cell cycle phases were determined using the fluorescent DNA dye propidium iodide (final concentration, 5 μg/mL; Molecular Probes) at 630 nm fluorescence emission (11).

Cell Death Analysis

Apoptotic and necrotic cell death were distinguished by using fluorescence microscopy (12). For this purpose, isolated cells were incubated with Hoechst 33342 (Molecular Probes; 10 μmol/L; which stains all nuclei) and propidium iodide (10 μmol/L; which stains nuclei of cells with a disrupted plasma membrane) for 3 min and analyzed using a Diaphot 300 fluorescence microscope (Nikon) with excitation at 360 nm. Nuclei of viable, necrotic, and apoptotic cells were observed as blue round nuclei, pink round nuclei, and fragmented blue or pink nuclei, respectively. About 1,000 cells were counted each time. DNA strand breaks in apoptotic cells were assayed by using a direct TUNEL labeling assay (Boehringer) and fluorescence microscopy following the manufacturer’s methodology.

Transfection of Green Fluorescent Protein

Long-term, stable expression of green fluorescent protein (GFP) in HT-29 cells was based on a previously described methodology (13). Briefly, 24 h before transfection, HT-29 cells were seeded in a six-well tissue culture plate at a density of 5 × 10⁵ in 2 mL growth medium and incubated overnight. On the day of transfection, plasmid DNA (geneticine-resistant pEGFP-C1; Clontech) was diluted into Opti-MEM (Invitrogen) and mixed with LipofectAMINE 2000 (Invitrogen) according to supplier’s protocol. Before transfection, the growth medium was replaced with 2 mL Opti-MEM. DNA-LipofectAMINE 2000 complexes were added to the cells and incubated for 6 h. The transfection medium was then replaced by growth medium and cells were incubated for an additional 18 h period. High-Performance Cell Sorting (DAKO) was used to select geneticine-resistant HT-29 clones expressing the GFP (HT-29-GFP) and showing high fluorescence emission.

Tumor Xenografts

For HT-29 cancer cell xenograft experiments, female nu/nu nude mice (ages 6–8 weeks; Charles Rivers Laboratories) were inoculated s.c. with 5 × 10⁶ HT-29 or HT-29-GFP cells per mouse. Tumor volume was calculated based on two dimensions, measured using calipers, and was expressed in cubic millimeters according to the formula: \( V = 0.5a \times b^2 \), where \( a \) and \( b \) are the long and short diameters of the tumor, respectively. For histologic analysis, the surgical and xenograft tissue samples were fixed in 4% formaldehyde, paraffin embedded, and stained with H&E and safran. Mice were monitored for at least 30 days after inoculation, and tumor measurements were taken on days 5, 10, 15, 20, 25, and 30. This study was conducted in compliance with international laws and policies (EEC Directive 86/609, OJ L 358. 1, December 12, 1987, and NIH Guide for the Care and Use of Laboratory Animals, NIH Publication 85-23, 1985).

Laser Microdissection

Excised HT-29-GFP tumor samples were embedded in freezing medium OCT (Tissue-Tek, Electron Microscopy Sciences) and immediately flash-frozen using isopentane and following Leica Microsystems instructions to preserve RNA. Tissue slices (5 μm) were obtained using a Leica 2800E Frigocut Cryostat Microtome. Tumor cells were separated using a Leica LMD6000 Laser Microdissection System equipped with an automated fluorescence module.

Reverse Transcription-PCR and Detection of mRNA Expression

Total RNA was isolated using the Trizol kit from Invitrogen and following manufacturer’s instructions.

4 www.orgsyn.org
cDNA was obtained using a random hexamer primer and a MultiScribe Reverse Transcriptase kit as described by the manufacturer (TaqMan RT Reagents; Applied Biosystems). A PCR master mix and AmpliTaq Gold DNA polymerase (Applied Biosystems) were then added containing the specific primers (Sigma-Genosys):

- bax (F-CCAGCTGGCGTGAAGGCCAGAC and R-ACCCCTCTCAAGACCTCTC),
- bak (F-TGAAAAATGCTCCTCGGCCAGAC and R-TCATGATTGGAAAGTCTCCTGCC),
- bad (F-AGGCGCTTACAGTTCC and R-CTGAGCGCAACGTCATC),
- bid (F-GCCTCCAGTGTAGACCGAGC and R-CTGCA-TCTGTTGGATGAT),
- bik (F-ATTTCGAGGTCCCTGCCGAG and R-GGCTT-CTCAGCTC),
- bim (F-GCCCCCTTACCTCCCATACAGC and R-CAGG-ATTCTCAGTTCTCGT),
- bcl-2 (F-CTCGTCGATTGGCCAGCTCTCC and R-CAAGTGGGGTCTGACTTCGAC),
- bcl-1 (F-CTGTCGATCGCTCTGACTTCGAC and R-CAAGTGGGGTCTGACTTCGAC),
- bcl-x (F-CTGTCGATCGCTCTGACTTCGAC and R-CAAGTGGGGTCTGACTTCGAC),
- Superoxide dismutase 1 (SOD1; F-TGAAAGGTGTGGGAAGCATT and R-TTACACCAAGCCCAAGGAC),
- Superoxide dismutase 2 (SOD2; F-CTGAGCCACACAAGCTCAGC and R-GTCACTCCTTCCTGAC),
- Catalase (F-CGAGAGAAGAGGGTAGTCAAG and R- AAACCTATTTTTTCCCTGG),
- Glutathione peroxidase (F-CTGGTGCTGGCTCTGCAAC and R-CAATGCTGGAAGCGGCCCCG),
- Glutathione reductase (F-GTCCACGCTTAGGAATAC- CAG and R-GTACTCCATGTCCAGTTCT),
- Thioredoxin reductase-1 (F-CTCGAGATATGCTCAGTCC and R- CATAGTACACTGTAACTGGT), and
- Glyceraldehyde-3-dehydrogenase (F-CTCTGAGAAGC- TGCCAGTAGT and R-GGCTCAGGGTGAAGCTCAGT).

Real-time quantitation of the mRNA relative to glyceralde-hyde-3-dehydrogenase was done with a SYBR Green I assay and a iCycler detection system (Bio-Rad). Target cDNA was amplified as follows: 10 min at 95°C then 40 cycles of amplification (denaturation at 95°C for 30 s and annealing and extension at 60°C for 1 min per cycle). The increase in fluorescence was measured in real-time during the extension step. The threshold cycle (C_t) was determined, and the relative gene expression was expressed as fold change = 2^{\Delta(C_{T})}, where \Delta C_T = C_T target - C_T glyceraldehyde-3-dehydrogenase and \Delta(\Delta C_T) = \Delta C_T treated - \Delta C_T control.

**Ionizing Radiation**

X-rays were administered using a 6 keV SL75 linear accelerator from Philips. For this purpose, each mouse was anesthetized with nembutal (50 mg/kg i.p.) and fixed on a Perspex platform. Single-fraction radiotherapy was admin-istered at a rate of 2 Gy/min and the radiation beam was focused only on the tumor. The irradiated area was fixed to a maximum of 1.2 cm², and the rest of the mouse had lead protection.

**Enzyme Assays**

Tumor tissue was homogenized in 0.1 mol/L phosphate (pH 7.2) at 4°C. Superoxide dismutase activity was measured as described by Flohe and Otting (14) using 2 mmol/L cyanide in the assay medium to distinguish manganese-type enzyme (SOD2) from the cuprozinc type (SOD1).

**Antisense Oligodeoxynucleotides**

Fully phosphorylated 21-mer human SOD2 antisense oligodeoxynucleotide (SOD2-AS) was obtained from Sigma-Genosys (sequence: 5’-GGAACCAACACAAAGC-GCA-3’). As a control, an equivalent but reversed phosphorylated 21-mer sequence was purchased from the same source.

SOD2-AS was loaded onto the lipid surface of cationic gas-filled microbubbles by ion charge binding as described previously (15). In vivo, uptake of a digoxigenin-labeled SOD2-AS was found in HT-29 tumor xenografts in nude mice following intratumoral injection of loaded microbubbles and subsequent exposure of the tumor to ultrasound (15).

Inhibition of SOD2 expression was verified by measuring the SOD2 activity and Western blot analysis. Tissue extracts were made by homogenization in a buffer containing 150 mmol/L NaCl, 1 mmol/L EDTA, 10 mmol/L Tris-HCl, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, 1 μg/mL aprotinin, and 1 μg/mL pepstatin (pH 7.4). Protein [50 μg; as determined by the Bradford assay (16)] were boiled with Laemmli buffer and resolved in 12.5% SDS-PAGE. Proteins were transferred to a nitrocel- lulose membrane and subjected to Western blotting with anti-human SOD2 monoclonal antibody (Sigma). Blots were developed using horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence (ECL System; Amersham).

**bcl-2 Gene Transfer and Analysis**

The Tet-Off gene expression system (Clontech) was used, as reported previously (17), to insert the human bcl-2 gene and for transfection into HT-29 cells following manufacturer’s instructions. Bcl-2 protein was quantitated in the soluble cytosolic fraction by enzyme immunoassay (17) using a monoclonal antibody-based assay from Sigma (1 unit of Bcl-2 was defined as the amount of Bcl-2 protein in 1,000 nontransfected HT-29 cells).

**Nuclear Factor-κB DNA Binding**

Evaluation of nuclear factor-κB (NF-κB) p50/65 DNA-binding activity in nuclear extracts of HT-29 cell samples was carried out to measure the degree of NF-κB activation. Nuclear extract were prepared as described previously (18). An ELISA was done in line with the manufacturer’s protocol for a commercial kit (Chemiluminescent NF-κB p50/65 Transcription Factor Assay, Oxford Biomedical Research). Briefly, this chemiluminescence-based sandwich-type ELISA employs an oligonucleotide, containing
the DNA-binding NF-κB consensus sequence, bound to a 96-well ELISA plate. NF-κB present in the sample binds specifically to the oligonucleotide coated on the plate. The DNA-bound NF-κB is selectively recognized by the primary antibody, which, in turn, is detected by the secondary antibody-alkaline phosphatase conjugate. Antibodies anti-cyclin D1 were used as negative controls.

**Immunocytochemical Detection of NF-κB p65**

HT-29 cells were grown in chamber slides and fixed with acetone. After two brief washes with PBS, slides were blocked with 5% normal goat serum for 1 h and then incubated with mouse monoclonal antibody anti-human p65 (Santa Cruz Biotechnology). After overnight incubation, the slides were washed and then incubated with rabbit anti-mouse IgG-Alexa 594 (Molecular Probes) for 1 h and counterstained with Hoechst stain (50 ng/mL) for 5 min. Stained slides were analyzed using a TCS-SP2 confocal microscope (Leica Microsystems).

**Western Blot Analysis of IκBα and Phosphorylated IκBα**

Whole-cell extracts were made by freeze-thaw cycles in buffer containing 150 mmol/L NaCl, 1 mmol/L EDTA, 10 mmol/L Tris-HCl, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, 1 μg/mL aprotonin, and 1 μg/mL pepstatin (pH 7.4). Protein [50 μg; as determined by the Bradford assay (16)] was boiled in Laemmli buffer and subjected to Western Bradford assay (16) was boiled in Laemmli buffer and subjected to Western

**Measurement of O2− and H2O2 Generation**

O2− generation was determined by flow cytometry using dihydroethidium (2 μg/mL; Molecular Probes). For this purpose, cellular suspensions were diluted to 200,000 fresh isolated leukocytes samples were suspended in 50 μL PBS containing 5% FCS and 0.1% sodium azide. Samples containing 5 × 10^5 cells were incubated in PBS + 5% FCS + 0.1% sodium azide with rat anti-mouse CD3 (clone KT3), rat anti-mouse CD4 (clone KT15) follows by streptavidin Cy5 coupled to R-phycocerythrin (DAKOCytomation) for 45 min on ice. Staining dot-blot analysis was done using a FACScan (Becton Dickinson). R-phycocerythrin-conjugated anti-NK1.1 (clone PK136) antibodies were used in double staining with anti-mouse CD3 labeled with FITC. Anti-mouse-κ for detection of κ-positive B cells was labeled with biotin (Southern Biotechnology Associates) and detected with streptavidin-FITC (Jackson Immuno-Research). Side scatter and forward scatter of dot plots were used to determine the gates of lymphocytes; R-phycocerythrin- or FITC-labeled IgG (PharMingen) served as isotype controls for R-phycocerythrin- or FITC-labeled antibodies. Fluorescence-activated cell sorting analysis was done using FACSCalibur flow cytometer (Becton Dickinson). Data were analyzed using CellQuest (Becton Dickinson).

**Statistical Significance**

Data were analyzed by Student’s t test.

**Results**

**In vitro Inhibition of HT-29 Growth by t-PTER and QUER**

Recently, we showed that in vitro growth of B16M-F10 cells was inhibited (~56%) by short time exposure (60 min/d) to PTER (40 μmol/L) and QUER (20 μmol/L) [approximate mean of the plasma concentrations measured within the first hour after i.v. administration of 20 mg of each polyphenol/kg (6)]. As shown in Fig. 1A, when this strategy was used against HT-29 human colorectal cancer
cells, the combination of t-PTER and QUER inhibited tumor growth in vitro (5 days after seeding) to ~52% of control values (~47% and 35% of the cells accumulated in G2-M and S phases, respectively, whereas controls growing exponentially showed a cell cycle distribution of ~50% in G0/G1, 26% in S, and 24% in G2-M; n = 7 in both cases; data not shown). Cell death analysis revealed that, 5 days after seeding, most nonviable cells (Fig. 1B) were apoptotic (>90% in all cases; data not shown).

**PTER- and QUER-Induced Growth Inhibition of HT-29 Xenografts**

Our next step was to investigate the effect of t-PTER and QUER on HT-29 colorectal cancer growth under in vivo conditions. As shown in Fig. 2, i.v. administration of t-PTER and QUER (20 mg of each polyphenol/kg × day, administered every day at 10:00 a.m.; dissolved as reported previously; see ref. 6), inhibited colorectal cancer growth to ~49% of control values (a percentage of inhibition that is coherent with the results reported in Fig. 1 under in vitro conditions). A lower dose (10 mg of each polyphenol/kg × day) inhibited colorectal cancer growth to ~87% of control values (n = 10; P < 0.05), whereas with a higher dose (30 mg of each polyphenol/kg × day), the percentage of colorectal cancer growth inhibition, compared with controls, was not significantly different to that found by administering 20 mg of each polyphenol/kg × day (n = 10 for each dose; data not shown). Therefore, in vivo administration of t-PTER and QUER, at clinically relevant doses, had a significant effect on human colorectal cancer growth.

t-PTER and QUER are pharmacologically safe because they have no organ-specific or systemic toxicity (including tissue histopathologic examination and regular hematology and clinical chemistry data) even when administered i.v. at a high dose (e.g., 30 mg of each polyphenol/kg × day × 23 days).5 However, although natural polyphenols may be used to sensitize tumor cells to chemotherapy and/or radiotherapy (20), the precise tumor resistance mechanisms influenced by natural polyphenols need to be defined under in vivo conditions.

**Gene Expression Profile of Bcl-2-Related Proteins and Antioxidant Enzymes in Polyphenol-Resistant HT-29 Cells**

Natural polyphenols can regulate expression of apoptosis regulators (7). Bcl-2 family proteins are regulators of chemoresistance and radioresistance in cancer (21–23). Besides, enhanced antioxidant mechanisms in tumor cells

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5 Estrela et al., unpublished results.
Table 1. Effect of natural polyphenols and chemoradiotherapy on HT-29 xenografts growth

<table>
<thead>
<tr>
<th>Tumor volume (mm³)</th>
<th>Physiologic saline</th>
<th>-</th>
<th>+</th>
<th>t-PTER + QUER</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physiologic saline</td>
<td>1,872 ± 344</td>
<td>1,097 ± 201*</td>
<td>1,140 ± 263†</td>
<td>530 ± 178†</td>
<td></td>
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<tr>
<td>FOLFIRI</td>
<td>643 ± 175</td>
<td>174 ± 60*</td>
<td>316 ± 91†</td>
<td>77 ± 41††</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOLFOX6</td>
<td>410 ± 106</td>
<td>145 ± 69†</td>
<td>70 ± 33†</td>
<td>ND</td>
<td></td>
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</table>

NOTE: Tumor volume 1 wk after inoculation was, in all cases, of 50 to 70 mm³; t-PTER and QUER (20 mg each/kg × day × 23 d, starting 1 wk after tumor inoculation) were administered i.v. Irinotecan (50 mg/kg) + leucovorin (120 mg/kg) + 5-fluorouracil (120 mg/kg; FOLFIRI) or oxaliplatin (30 mg/kg) + leucovorin (120 mg/kg) + 5-fluorouracil (120 mg/kg; FOLFOX6) were administered i.v. on days 25 and 28 after tumor inoculation. Animal doses of chemotherapy were calculated using the National Cancer Institute human recommended doses for each drug (www.fda.gov) and the conversion factor for mice published by the Food and Drug Administration (Center for Drug Evaluation and Research; www.fda.gov). Mice received fractionated X-ray therapy (5 Gy/d focused on the tumor irradiation area was 1.0-1.2 cm²) on days 22 and 24 after tumor inoculation [below maximum tolerated doses because the LD₅₀ reported for mice subjected to whole body irradiations is ~7.5 to 8 Gy (e.g., ref. 62)]. Tumor volumes displayed in the table refer to those measured 30 d after inoculation. Mean ± SD of 12 to 15 mice per group. ND, nondetectable. Histologic examination (see Xenografts in Materials and Methods) confirmed that, in 17 of 20 (~85%) mice, the full treatment achieved a complete tumor regression. The significant test refers to the comparison between X-ray treatment and nonirradiated mice (†P < 0.05, ††P < 0.01) and between t-PTER + QUER administration and treatments without polyphenols (‡P < 0.05, ‡‡P < 0.01).
amino transferase, γ-glutamyl transpeptidase, alkaline phosphatase, and lactate dehydrogenase. However, in the mice cleared of tumor (85%, >120-day survival; see above), all hematologic and clinical chemistry measures practically returned to normal values measured in untreated, non-tumor-bearing mice by 30 days after treatment (Table 2).

**SOD2 and Bcl-2 Are Key Targets in the Mechanism Activated by the Polyphenol Association**

Different mechanisms can influence colorectal cancer growth and/or survival (see above). Following the findings displayed in Fig. 3, SOD2 and Bcl-2 were selected to investigate their role in the increased drug and radiation antitumor efficacy found in combination with t-PTER and QUER. For this purpose, we used two different strategies: intratumoral injection of a SOD2-AS to decrease the SOD2 activity in growing HT-29 cells and genetic engineering to obtain bcl-2-overexpressing HT-29 cells (HT-29/Tet-bcl-2). As shown in Table 3A, treatment of HT-29-bearing mice with t-PTER and QUER increased SOD2 activity (without affecting the SOD1). Besides, treatment of HT-29-bearing mice with the polyphenol association decreased Bcl-2 levels (Table 3A). Treatment with SOD2-AS decreased the SOD2 activity but without affecting Bcl-2 levels (Table 3A). In HT-29/Tet-bcl-2 cells, bcl-2 overexpression (compared with HT-29 control cells) was the only difference (Table 3A). Changes in SOD2 activity or Bcl-2 levels displayed in Table 3A affect the Bcl-2 levels (Table 3A). Treatment with polyphenols and chemoradiotherapy was significantly different (Table 3B). However, combined treatment with polyphenols and chemoradiotherapy was unable to induce a complete tumor regression in HT-29/Tet-bcl-2 xenografts or in HT-29 xenografts treated with the SOD2-AS (Table 3B). These facts prove that SOD2 up-regulation and Bcl-2 down-regulation facilitate the complete colorectal cancer regression reached by combination of polyphenols with chemoradiotherapy.
**Polyphenols Down-regulate bcl-2 Expression by Inhibiting NF-κB Activation**

NF-κB contributes to development and/or progression of malignancy by regulating the expression of genes involved in cell growth and proliferation, antiapoptosis, angiogenesis, and metastasis (28). NF-κB may inhibit apoptosis in colorectal cancer cells through activation of expression of antiapoptotic genes, such as bcl-2 (29). In fact, inactivation of NF-κB in different cancer cells has been shown to blunt the ability of the cancer cells to grow (30).

Some reports suggested that natural polyphenols (e.g., the green tea constituent epigallocatechin 3-gallate) inhibit growth, in part, through blocking of the signal transduction pathways leading to activation of critical transduction factors such as NF-κB (31). Thus, we investigated if t-PTER- and QUER-induced down-regulation of bcl-2 was linked to the mechanism of NF-κB activation. As shown in Fig. 4A, polyphenols decreased binding of NF-κB to the DNA compared with controls [we found a linear correlation (r² > 0.99) between relative light units and amount of NF-κB]. Total cell extracts from HT-29 cells cultured in the presence of tumor necrosis factor-α (TNF-α) served as positive control (Fig. 4). Suppression of NF-κB activation was also confirmed by immunocytochemistry, because t-PTER and QUER inhibited nuclear translocation of p65 in HT-29 cells immunostained with antibody anti-p65 and then visualized with Alexa 594-conjugated second antibody (see Materials and Methods; data not shown).

Whether inhibition of NF-κB activation was due to inhibition of IkBa (the most prominent member of the IkB family in mammalian cells) degradation was examined next. As shown in Fig. 4, polyphenols also inhibited IkBa degradation (Fig. 4B) and, in parallel, decreased the content of phosphorylated IkBa (Fig. 4C).

To investigate further if inhibition of NF-κB is fully or partially responsible of down-regulating bcl-2 expression, cultured HT-29 cells were treated with NF-κB p65-specific siRNA. Western blot analysis showed that p65 siRNA depletes the intracellular content of the protein (Fig. 5A). Whereas p65 siRNA or dehydroxymethylepoxyquinomicin (which specifically inhibits nuclear translocation and

<table>
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<tr>
<th>Table 3. Effect of SOD2 silencing and/or bcl-2 overexpression on HT-29 cell resistance to treatment with natural polyphenols and chemoradiotherapy in vivo</th>
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<tr>
<td>units/mg protein</td>
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<tr>
<td></td>
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<tr>
<td>HT-29</td>
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<tr>
<td></td>
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<tr>
<td>HT-29/Tet-Bcl-2</td>
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<td></td>
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<tr>
<td>−SOD-AS −PQ +PQ −SOD-AS +SOD-2-AS −PQ +PQ −SOD-AS +SOD-2-AS</td>
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<td>SOD2  1.35 ± 0.23  4.9 ± 0.56  0.27 ± 0.05  1.18 ± 0.24  1.12 ± 0.3  5.6 ± 0.64  0.33 ± 0.12  1.06 ± 0.25</td>
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<td>SOD1  6.24 ± 0.47  6.84 ± 0.83  5.77 ± 0.62  6.05 ± 0.75  5.49 ± 0.39  6.17 ± 0.77  6.3 ± 0.48  5.87 ± 0.95</td>
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<td>Bcl-2  21 ± 3  6 ± 2  19 ± 3  7 ± 1  75 ± 6  53 ± 5  81 ± 9  57 ± 6</td>
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**A**

| Physiologic saline | 1,645 ± 317 | 1,860 ± 412 |
| Chemoradiotherapy + PQ | ND<sup>3</sup> | 217 ± 71<sup>3</sup> |
| SOD2-AS | 2,337 ± 266<sup>3</sup> | 2,942 ± 387<sup>3</sup> |
| Chemoradiotherapy + PQ + SOD2-AS | 167 ± 46<sup>3</sup> | 584 ± 129<sup>3</sup> |

**B**

<table>
<thead>
<tr>
<th>Tumor volume (mm&lt;sup&gt;3&lt;/sup&gt;)</th>
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<tbody>
<tr>
<td>HT-29</td>
</tr>
<tr>
<td>HT-29/Tet-Bcl-2</td>
</tr>
<tr>
<td>Physiologic saline</td>
</tr>
<tr>
<td>Chemoradiotherapy + PQ</td>
</tr>
<tr>
<td>SOD2-AS</td>
</tr>
<tr>
<td>Chemoradiotherapy + PQ + SOD2-AS</td>
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*NOTE: Mice were inoculated with cultured HT-29 or HT-29/Tet-bcl-2 cells. PQ: t-PTER + QUER. (A) SOD2 and SOD1 activities and Bcl-2 levels in HT-29 and HT-29/Tet-bcl-2 xenograft samples obtained from control or from tumor-bearing mice treated with t-PTER + QUER (20 mg each/kg of body weight, as indicated in Fig. 2) and/or SOD2-AS (5 mg/kg body weight each 3 d, starting 4 d after tumor inoculation). Intratumoral injection of SOD2-AS was done as explained in Materials and Methods. A reversed-sequence control SOD2-AS was used for comparison, but results were not significantly different from those obtained in physiologic saline-treated mice (data not shown). Histopathologic examination of the xenograft samples revealed that most tissue (>95% in all cases) corresponds to tumor cells. Mean ± SD of 8 to 10 mice per group. (B) HT-29-bearing or HT-29/Tet-bcl-2-bearing mice were treated with t-PTER + QUER, chemoradiotherapy (FOFOX6 and X-rays; as indicated in Table 1), and SOD2-AS (as indicated above). Tumor volume was measured 30 d after inoculation. Mean ± SD of 10 to 12 mice per group. 

<sup>1</sup>P < 0.01 comparing treatment with t-PTER + QUER (PQ) versus treatment with PS.

<sup>2</sup>P < 0.01 comparing treatment with SOD2-AS versus treatment with phosphorylase b.

<sup>3</sup>P < 0.01 comparing all conditions versus physiologic saline-treated controls.

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activation of NF-κB; ref. 30) induced a significant decrease in NF-κB binding to DNA (Fig. 5B) and in bcl-2 (Fig. 5C) expression, which is similar to the decrease reported in Fig. 3 in HT-29 cells growing in mice treated with t-PTER and QUER. Therefore, our results indicate that t-PTER- and QUER-induced down-regulation of bcl-2 is NF-κB dependent.

Polyphenols Up-regulate SOD2 Expression via a SP1-Dependent Mechanism

Transcriptional activation of human SOD2 mRNA, induced by t-PTER and QUER (Fig. 3), was examined to identify the responsive transcriptional regulator. Based on the results shown above, t-PTER- and QUER-induced up-regulation of SOD2 (Fig. 3) should involved a NF-κB-independent mechanism.

Computer analysis and foot-printing assays revealed several putative binding sites for SP1 and AP2 transcription factors in the proximal promoter of human SOD2. These two proteins are main transcriptional regulators of human SOD2 expression but appear to have opposite effects: whereas the SP1 element positively promotes transcription, the AP2 proteins significantly repress the promoter activity (32). Both SP1 and AP2 are expressed in HT-29 cells (33). To

Figure 4. t-PTER and QUER inhibit NF-κB. HT-29 cells were cultured and treated with polyphenols as indicated in Fig. 1. A, NF-κB binding to DNA (measured 3 h after the last polyphenol addition). Recombinant human TNF-α (0.5 nmol/L; Sigma) was added 6 h before the last polyphenol addition. lane 1, TNF-α; lane 2, control; lane 3, t-PTER + QUER. Relative densitometric intensities for protein bands from Western blots of IkBα and phosphorylated IkBα were normalized to β-actin (black column, TNF-α; white column, control; gray column, t-PTER + QUER). Mean ± SD of 5 different experiments in each experimental condition. *, P < 0.01, comparing each value versus controls (where basal medium was added instead of polyphenols and/or TNF-α).

Figure 5. Effect of siRNA-induced NF-κB p65 depletion on NF-κB binding to DNA and bcl-2 expression. Transfection of siRNA was done as explained in Materials and Methods. A, Western blot analysis of p65 in cells transfected with p65 or NS siRNA. For comparison, NF-κB binding to DNA (B) and bcl-2 expression (RT-PCR; C) were determined in HT-29 cells transfected with p65 siRNA or treated with dehydroxymethylepoxyquinomicin (DHMEQ). For this purpose, cultured HT-29 cells (24 h after seeding) were incubated in the absence or in the presence of 10 μg DHMEQ/mL, and NF-κB activation and bcl-2 expression were measured at 2 and 12 h, respectively, after removing the inhibitor. Mean ± SD of 4 to 5 different experiments. *, P < 0.01, comparing all values versus controls.
answer if t-PTER- and QUER-induced increased expression of SOD2 is mediated by these transcriptional regulators, we treated cultured HT-29 cells with t-PTER + QUER and SP1- or AP2-specific siRNA. Western blot analysis shows that SP1 and AP2 siRNAs deplete the intracellular content of their corresponding proteins (Fig. 6A and B). However, as shown in Fig. 6C, t-PTER- and QUER-induced up-regulation of SOD2 expression appears mainly dependent on SP1.

Discussion

The low bioavailability of t-RESV (5) prompted us to test the anticancer activity of other structurally related molecules. t-PTER, a natural analogue of t-RESV but 60 to 100 times stronger as an antifungal agent, shows similar anticarcinogenic properties (34), whereas QUER may affect tumor cell proliferation and targets key molecules responsible for tumor cell properties, including p53 and oncogenic Ras (35–37).

Bioavailability and in vivo biological efficacy must be correlated before drawing conclusions on potential health benefits of polyphenols. Phenolic compounds are potential inhibitors of growth and promote apoptosis in different human colorectal cancer cell lines (e.g., ref. 38). Nevertheless, although natural polyphenols appear to decrease chemically induced colon carcinogenesis in different experimental models (e.g., ref. 39), it is not known if their systemic administration would affect colorectal cancer growth in vivo. In fact, compounds such as curcumin are effective when applied topically to the skin or administered orally to affect the colon but have not been shown effective in internal organs such as the lungs (39).

We show that short time exposure (60 min/d) to bioavailable concentrations of t-PTER and QUER (6) inhibited in vitro growth of HT-29 cells by ~48% (Fig. 1A), whereas viability of the remaining cells decreased to ~71% (>95% in controls; Fig. 1B). Loss of cell viability was mainly due to apoptosis. In fact, Tinhofer et al. (40) suggested a direct interaction of t-RESV with mitochondria triggering the loss of mitochondrial membrane potential and the opening of the Bcl-2-sensitive pore, and we have shown that t-PTER and QUER induce a nitric oxide-dependent inhibition of bcl-2 expression in metastatic cells, thus facilitating the tumor cytotoxicity elicited by the endothelium (6, 41). I.v. administration of t-PTER and QUER (20 mg of each polyphenol/kg × day) also inhibited HT-29 xenograft growth to ~49% of control values (Fig. 2B). The association of t-PTER and QUER induced a stronger inhibition of colorectal cancer growth than each polyphenol alone (Fig. 2). I.v. administration of 40 mg t-PTER or QUER/kg × day (n = 5 in each case; data not shown) induced a colorectal cancer growth inhibition, which was not significantly different to that shown for the 20 mg/kg × day dose, thus indicating that the association, and not a higher dose of one, gives better results. In fact, in the B16 melanoma model, t-PTER increases the expression of pro-death BAX and decreases expression of anti-death Bcl-2, whereas QUER increases the expression of all pro-death genes analyzed (BAX, BAK, BAD, and BID) and decreases the expression of all anti-death genes analyzed (Bcl-2, Bcl-w, and Bcl-xL; ref. 6). Therefore, it appears plausible to expect benefits when using the combination.

In vivo treatment with t-PTER and QUER altered expression of molecules involved in regulating cancer cell resistance to drugs and radiations (e.g., the Bcl-2 family of pro-death and anti-death proteins and the antioxidant enzyme system; Fig. 3). Multidrug and/or radiation resistance are characteristic features of malignant tumors, and in practice, intrinsic (innate) or acquired (adaptive) resistance to therapy critically limits the outcome of cancer patients (42).

The proto-oncogene bcl-2 and its antiapoptotic homologues are mitochondrial membrane permeabilization inhibitors (43) and participate in the development of chemoresistance (21), whereas expression of pro-death genes (e.g., bax or bak) is often reduced in cancer cells (44). As shown in Fig. 3, treatment with t-PTER and QUER significantly increased expression of the proapoptotic genes bax, bak, bad, and bid (1.9- to 2.5-fold) but decreased that of the antiapoptotic bcl-2 (3.3-fold). This is important because down-regulation of bcl-2 expression can lead to chemoresistance of carcinoma cells (e.g., ref. 45), and we have shown that antisense oligodeoxynucleotide-induced specific depletion of Bcl-2 facilitates regression of malignant melanoma in mice treated with chemotherapy and ionizing radiations (23).

Reactive oxygen species, acting as intracellular second messengers, promote proliferation and maintain the oncogenic phenotype of cancer cells (46). Moreover, reactive

Figure 6. Effect of siRNA-induced SP1 and AP2 depletion on the induction of SOD2 expression by t-PTER and QUER. Transfection of siRNA was done as explained in Materials and Methods. HT-29 cells were cultured and treated with polyphenols as indicated in Fig. 1. Western blot analysis of SP1 (A) and AP2 (B) in cells transfected with SP1 siRNA (A), AP2 siRNA (B), or NS siRNA and treated with polyphenols. Western blots displayed in A and B were found similar either before or after treatment with polyphenols (data not shown). SOD2 expression was analyzed by real-time quantitative-PCR (see Materials and Methods) 6 h after the last addition of t-PTER and QUER. Data, expressing fold change, show mean ± SD of 5 to 6 different experiments. *, P < 0.01, comparing all data versus control values in the absence of t-PTER + QUER treatment.
oxygen species control the expression of Bcl-2 family proteins by regulating their phosphorylation and ubiquitination (47). A recent report shows that very low concentrations of QUER and rutin (0.1-1 μmol/L) decrease expression of SOD1 and increase that of glutathione peroxidase, thus diminishing reactive oxygen species (48). However, i.v. administration of t-PTER and QUER (Fig. 3) increased expression of SOD1 (~1.6-fold), SOD2 (5.7-fold), and catalase, glutathione peroxidase, glutathione reductase, and thioredoxin reductase-1 (~2-fold). As shown in Fig. 7, these changes in antioxidant enzymes activities results in H₂O₂ accumulation compared with controls. A fact which appears in agreement with previous results showing that the tumor-suppressive effect of SOD2 overexpression is in part mediated by an antioxidant imbalance resulting in the reduced capacity to metabolize increased levels of intracellular peroxides (26).

Due to a higher production of superoxide anions by the respiratory chain and cytoplasmic NADPH oxidase, the basal concentration of reactive oxygen species (and particularly H₂O₂) is higher in cancer cells than in their normal counterparts (49). Thus, it is plausible that an increase in SOD2 activity, as reported here (Table 3A), could cause H₂O₂-induced cytotoxicity and decreased proliferation (ref. 49; see also Fig. 7 showing increased H₂O₂ generation). Huang et al. (50) suggested that malignant cells may be highly dependent on SOD for survival and proposed SOD activity as a possible target for the selective killing of cancer cells. Numerous in vivo studies show that eventually SOD can be highly expressed in aggressive human tumors and that high SOD activities have been associated with poor prognosis and resistance to cytotoxic drugs and radiation [see ref. 51 for a review]. However, SOD2 overexpression has been correlated in different cancer cell types, including colorectal cancer cells, with suppression of neoplastic transformation, decreased proliferation in vitro, and reversion of malignant phenotype (51). Uncoupling of the electrochemical gradient by increased SOD2 activity can give rise to p53 up-regulation and induction of senescence in colorectal cancer cells (52), whereas p53-induced suppression of bcl-2 expression can activate the mechanism of cell death (53). Nevertheless, HT-29 cells have a mutated p53 (54); although it may be relevant in other models, a link between SOD2 and p53 in HT-29 cells is unlikely.

As shown in Table 3, combination of t-PTER, QUER, chemotherapy, and radiotherapy eliminated HT-29 cells growing in vivo in most cases (85%; see Results and Table 3) leading to long-term survival (>120 days). However, as shown in Table 3, specific overexpression of bcl-2 and/or down-regulation of the SOD2 activity decreased the anti-cancer efficacy of polyphenols and chemoradiotherapy, thus proving that key molecules regulate resistance of colorectal cancer cells and may determine the efficacy of the therapy.

t-PTER- and QUER-induced down-regulation of bcl-2 expression involves polyphenol-induced inhibition of NF-κB activation and inhibition of IκBα phosphorylation and degradation (Fig. 4). Indeed, natural polyphenols (including t-RESV, epigallocatechin gallate, or quercetin) are known NF-κB inhibitors (55). Recently, we reported that t-PTER and QUER down-regulated inducible nitric oxide synthase, thus causing a nitric oxide shortage-dependent decrease in cyclic AMP response element-binding protein phosphorylation and a decrease in bcl-2 expression in B16M-F10 cells (41). Active NF-κB participates in the control of transcription of over 150 target genes, including inducible nitric oxide synthetase (56); thus, a decrease in endogenous nitric oxide generation may be also the link between inhibition of NF-κB activation (Fig. 4) and down-regulation of bcl-2 expression in HT-29 cells (Fig. 3). Nevertheless, this possibility needs experimental confirmation. On the other hand, a wide variety of stimuli can up-regulate SOD2 expression. The cytokine (interleukin-1, interleukin-4, interleukin-6, tumor necrosis factor-α, and IFN-γ) inducible enhancer regions contain binding sites for NF-κB, C/EBP, and NF-1 transcription factors, whereas protein kinase C stimulating agents, such as the phorbol ester 12-O-tetradecanoylphorbol-13-acetate, induce human SOD2 via a CREB-1/ATF-1-like factor but not via NF-κB or AP1 (57). Moreover, different microtubule-active anticancer drugs (e.g., paclitaxel or vincristine) may induce SOD2 expression via activation of protein kinase C and not via NF-κB (58). Here, we show that t-PTER and QUER, which inhibit NF-κB activation (Fig. 4), up-regulate SOD2 expression in human HT-29 cells via a SP1-dependent mechanism (Fig. 6). SP1 positively promotes transcription (32) and its decrease completely prevented the polyphenol-induced increase in SOD2 expression (Fig. 6).

Clinical applications may be derived from our study because chemotherapy and radiotherapy doses used are within clinical standards and because i.v. administration of t-PTER and QUER, at the doses here reported, appears safe (see Results). As reviewed by Lamson and Brignall (59), daily i.v. bolus doses of 100 mg QUER/m² were well tolerated by human patients showing no side effects or toxicity, whereas i.v. bolus of 1,400 mg QUER/m² (~2.5 g in a 70 kg adult) once weekly for 3 weeks was associated
with renal toxicity in 2 of 10 patients. The two patients had a reduction in glomerular flow rate of nearly 20% in the first 24 h. The reduction resolved within 1 week, and this effect was not cumulative over subsequent doses in the phase I trial in a population of advanced cancer patients. Transient flushing and pain at the injection site were noted in a dose-dependent manner. Therefore, the 1,400 mg QUER/m² × week dose was recommended for phase II trials.

I.v. doses of 100 mg/m² (~178 mg/d in a 70 kg adult) are equivalent to ~2.5 mg/kg × day. Therefore, 8 times lower than the dose of 20 mg/kg × day was used here. However, the U.S. Food and Drug Administration and the National Cancer Institute have indicated that extrapolation of animal doses to human doses is correctly done only through normalization to body surface area (60). Thus, the human dose equivalent (mg/kg) = animal dose (mg/kg) normalization to body surface area (60). Thus, the human doses to human doses is correctly done only through CancerInstitute have indicated that extrapolation of animal the U.S. Food and Drug Administration and the National than the dose of 20 mg/kg equivalent to 2.5 mg/kg/C2 day was used here. However, blueberries can inhibit colon cancer cell proliferation and induce apoptosis. J Agric Food Chem 2005;53:7320 – 9.


Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
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