

Dietary Curcumin Inhibits Chemotherapy-induced Apoptosis in Models of Human Breast Cancer¹

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ABSTRACT

Curcumin, the major component of the spice turmeric, is used as a coloring and flavoring additive in many foods and has attracted interest because of its anti-inflammatory and chemopreventive activities. However, this agent also inhibits the generation of reactive oxygen species (ROS) and the c-Jun NH₂-terminal kinase (JNK) pathway, and because many chemotherapeutic drugs generate ROS and activate JNK in the course of inducing apoptosis, we considered the possibility that curcumin might antagonize their antitumor efficacy. Studies in tissue culture revealed that curcumin inhibited camptothecin-, mechlorethamine-, and doxorubicin-induced apoptosis of MCF-7, MDA-MB-231, and BT-474 human breast cancer cells by up to 70%. Inhibition of programmed cell death was time and concentration dependent, but occurred after relatively brief 3-h exposures, or at curcumin concentrations of 1 μ M that have been documented in Phase I chemoprevention trials. Under these conditions, curcumin exhibited antioxidant properties and inhibited both JNK activation and mitochondrial release of cytochrome *c* in a concentration-dependent manner. Using an *in vivo* model of human breast cancer, dietary supplementation with curcumin was found to significantly inhibit cyclophosphamide-induced tumor regression. Such dietary supplementation was accompanied by a decrease in the activation of apoptosis by cyclophosphamide, as well as decreased JNK activation. These findings support the hypothesis that dietary curcumin can inhibit chemotherapy-induced apoptosis through inhibition of ROS generation and blockade of JNK function, and suggest that additional studies are needed to determine whether breast cancer patients undergoing chemotherapy should avoid curcumin supplementation, and possibly even limit their exposure to curcumin-containing foods.

INTRODUCTION

Curcuma longa Linn is a perennial herb originally cultivated widely in tropical regions of Asia from which dried rhizome is isolated the spice turmeric (reviewed in Refs. 1–3). Curcumin, also known as diferuloyl methane, is the major yellow pigment extracted from turmeric, which is used extensively in curries. Its properties as a coloring and flavoring agent have led to uses as a dietary additive in a variety of foods (1, 3, 4). These include saffron, mustard and other spices, gelatins, puddings, sorbets, ice creams, soups, meats, pickles, margarine, and both alcoholic and nonalcoholic beverages.³ Extracts containing curcumin have also been used in medicines in India and Southeast Asia for generations, and according to tradition are useful in the treatment of inflammation, skin wounds, hepatic and biliary disorders, cough, and coryza, as well as certain tumors (1, 3, 4). As a result, dietary intake of curcumin is especially high in these areas of Asia, where adults

consume up to ≥ 200 mg of curcumin/day or up to 7.8 μ mol/kg of body weight (4). Even in France, however, where curcumin exposure may be more representative of that typical worldwide, intake of as much as ≥ 3.4 μ mol/kg/day has been documented (5).

The exposure of populations worldwide to curcumin, and its many uses, has led to studies aimed at elucidating some of its activities. Curcumin and related compounds inhibit free radical generation and act as free radical scavengers (6–8) and antioxidants (9), inhibiting lipid peroxidation (10, 11) and oxidative DNA damage (12). Inhibition of lipoxygenase and cyclooxygenase (13) resulting in decreased arachidonic acid release and metabolism, along with abilities to inhibit activation of NF- κ B⁴ (14, 15), may contribute to the anti-inflammatory activity of these compounds. Another property ascribed to curcumin is that of inhibition of c-jun/AP-1 function (16) and JNK activation (17). Curcuminoids have been noted to be potent inhibitors of cytochrome P450 (18) and to have the ability to induce glutathione *S*-transferase (19), and as such, have been proposed as potential chemoprotective agents (reviewed in Ref. 20). Because curcumin inhibits tumor formation in several murine tissues and antagonizes both initiation and promotion of tumors in rodent epithelial and colon cancer models (1–3), interest has been raised in this compound as a chemopreventive agent (reviewed in Ref. 21). Most recently, curcumin has demonstrated antiangiogenic properties in several laboratory and *in vivo* model systems (22–24). These properties of curcumin have led to several Phase I human trials that have shown this agent to be tolerated well (25, 26), and their successful completion suggests that curcumin use may increase in the future.

Curcumin's chemopreventive activity in animal model systems has led investigators to study its potential impact upon tumor cell growth and apoptosis. Several reports document an antiproliferative effect on cultured cells such as on colon cancer (27) and breast cancer cells (28). This may, in part, be because of programmed cell death because at high concentrations curcumin can induce apoptosis such as in human leukemia cells (29). In contrast, in other systems curcumin can inhibit apoptosis such as in T lymphocytes (30), and it protected rat lungs from injury by bleomycin (31) and rat myocardium from Adriamycin (32), respectively, but its impact on the therapeutic applications of antineoplastic drugs has not been well studied. Because ROS have been felt to play important roles in drug-induced apoptosis (reviewed in Ref. 33), one might suspect that curcumin, as an antioxidant and free radical scavenger, would inhibit the ability of chemotherapeutic drugs to induce apoptosis. Furthermore, curcumin inhibits JNK activation (17), which has been associated with chemotherapy-mediated induction of apoptosis in tumor cells (reviewed in Ref. 34). We therefore considered the possibility that curcumin might decrease the effectiveness of antitumor drugs, and we used breast cancer as an example of the possible systemic effects of this dietary compound. Here we report, using both tissue culture and *in vivo* models of human breast cancer, that curcumin inhibited the

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⁴The abbreviations used are: NF- κ B, nuclear factor κ B; AP-1, activator protein-1; JNK, c-Jun NH₂-terminal kinase; ROS, reactive oxygen species; LCCC TCF, Lineberger Comprehensive Cancer Center Tissue Culture Facility; ssDNA, single-stranded DNA; SAPK, stress-activated protein kinase.

proapoptotic activity of several chemotherapeutic agents and also inhibited ROS generation, JNK activation, and release of mitochondrial cytochrome *c*. These findings support the need for further study into the effects of dietary curcumin on patients receiving breast cancer chemotherapy, and may indicate a need for such patients to avoid dietary supplementation with curcumin and perhaps even limit their intake of foods containing this agent.

MATERIALS AND METHODS

Chemicals. Curcumin, mechlorethamine, and cyclophosphamide were from Sigma Chemical Co. (St. Louis, MO), whereas camptothecin and doxorubicin were obtained from Calbiochem-Novabiochem Corp. (San Diego, CA). Stock solutions were prepared in 95% ethanol (mechlorethamine; Mallinckrodt Baker, Inc., Paris, KY), PBS (cyclophosphamide; LCCC TCF), or DMSO (curcumin, camptothecin, and doxorubicin) and stored at -20°C . These agents were added to the concentrations indicated, with a final vehicle concentration of $\leq 0.5\%$ (v/v). Aprotinin and leupeptin were from Roche Molecular Biochemicals (Indianapolis, IN). All other chemicals used were from Fisher Scientific (Fair Lawn, NJ).

Cell Lines and Cell Culture. MCF-7, MDA-MB-231, and BT-474 human breast carcinoma cells (LCCC TCF) were propagated in Richter's modified Eagle's medium supplemented with insulin, gentamicin, and 20 mM HEPES (LCCC TCF), and 9% FCS, penicillin G sodium at 100 units/ml, and streptomycin sulfate at 100 $\mu\text{g}/\text{ml}$ (Life Technologies, Inc., Grand Island, NY).

Apoptosis Assays. To directly visualize apoptosis-associated DNA fragmentation, $3.0\text{--}4.0 \times 10^6$ cells subjected to conditions described in the text were detached, and their supernatants were collected by centrifugation. Pellets were resuspended in 10 mM Tris-HCl (pH 8.0), containing 10 mM EDTA and 0.5% Triton X-100, and lysed by vortexing, and debris was removed by centrifugation. After extraction with phenol-chloroform/isoamyl alcohol, nucleic acids were ethanol precipitated, collected by centrifugation, redissolved in 10 mM Tris-HCl (pH 8.0), containing 1 mM EDTA, 50 mM NaCl, and DNase-free RNase (Roche Molecular Biochemicals), and incubated at 37°C for 2 h. DNA separated in 1.5% agarose gels was visualized by staining with ethidium bromide.

For quantitative assessment of oligonucleosomal DNA fragmentation, medium containing the agents tested was added to 1.2×10^5 cells, and apoptosis was detected using the Cell Death Detection ELISA^{PLUS} kit (Roche Molecular Biochemicals). Spectrophotometric data at a wavelength of 405 nm, with a reference of 490 nm, were acquired on a MAXline Vmax kinetic microplate reader (Molecular Devices Corporation, Sunnyvale, CA). The enhancement of apoptosis was calculated in relation to control cells receiving vehicle alone and tabulated in KaleidaGraph version 3.0.1 (Synergy Software, Reading, PA).

To perform caspase 3 activity assays, 1.0×10^6 cells exposed to conditions detailed subsequently were harvested, collected by centrifugation, and lysed with 10 mM Tris-HCl (pH 7.5), containing 10 mM sodium phosphate, 135 mM NaCl, 1% Triton X-100, 10 mM Na PP_i, 1 mM phenylmethylsulfonyl fluoride, 1 $\mu\text{g}/\text{ml}$ aprotinin, and 1 $\mu\text{g}/\text{ml}$ leupeptin, for 10 min at 4°C . The lysates were clarified by centrifugation, aliquots were set aside for protein concentration determinations using the BCA assay (Pierce Chemical Co., Rockford, IL), and caspase 3 activity was evaluated using the substrate acetyl-aspartyl-glutamyl-valyl-aspartate (PharMingen, San Diego, CA). Release of the 7-amino-4-methyl-coumarin was determined by fluorescence spectrophotometry using an excitation wavelength of 380 nm, and an emission wavelength of 460 nm, and a Perkin-Elmer LS50B luminescent spectrometer (Perkin-Elmer Instruments, Shelton, CT). The ratio of caspase 3 activity was calculated in relation to control cells, which received vehicle alone.

ROS Assay. Cells (1.0×10^6) exposed to conditions detailed below subsequently were washed with ice-cold PBS, detached, collected, resuspended in PBS, and transferred to 96-well plates in triplicate. Fifty μl of a 16 mM solution of dichlorodihydrofluorescein diacetate (Sigma Chemical Co.) in PBS was then added, and the plates were incubated at 37°C for 1 h. Fluorescence at an emission wavelength of 485 nm, after excitation at 530 nm, was assayed as described above. An aliquot of the cell suspension was lysed and protein concentrations were determined. The final results were expressed as arbitrary absorbance units/mg protein.

JNK Assays. JNK activity, as judged by the presence of phosphorylated c-Jun protein, was determined with the Trans-AM. AP-1 c-Jun ELISA kit, which was used according to the manufacturer's specifications (Active Motif, Carlsbad, CA). Briefly, AP-1 heterodimeric complexes from nuclear extracts were captured by binding to a consensus 5'-TGA(C/G)TCA-3' oligonucleotide immobilized on a 96-well plate. The phospho-c-Jun content of the bound AP-1 was determined in a colorimetric reaction using a phospho-c-Jun primary antibody and a secondary horseradish peroxidase-conjugated antibody. Spectrophotometric data were expressed as a ratio of absorbance of each experimental condition compared with control cells exposed to vehicle alone.

Cytochrome *c* Release Assay. Cells (1.0×10^6) were exposed to conditions described in the text, collected, and homogenized on ice. The resultant lysate was clarified by centrifugation, and aliquots containing the cytosolic fraction were set aside for protein concentration measurements. Samples were mixed with 6 \times SDS sample buffer containing mercaptoethanol, denatured and reduced by heating, and subjected to SDS-PAGE. After separation, the proteins were electrophoretically transferred to nitrocellulose filters and subjected to Western blotting with murine monoclonal antibody 7H8.2C12 to cytochrome *c* (PharMingen). Immunoreactive protein bands were detected using the Phototope-Horseradish Peroxidase Western detection kit (Cell Signaling Technology, Inc.). To quantify protein bands, autoradiographs were scanned into Adobe Photoshop 5.0 (Adobe Systems, Inc., San Jose, CA), and densitometry was performed using NIH Image version 1.61 by laboratory members not involved in this project who were blinded to the experimental conditions.

Tumor Xenograft Modeling. All experiments were performed under a protocol approved by the University of North Carolina at Chapel Hill's Institutional Animal Care and Use Committee. *Mycoplasma*-free MCF-7 and BT-474 cells were injected s.c. in the flanks of *nu/nu* mice (Charles-River Laboratories, Inc., Wilmington, MA). Animals receiving MCF-7 cells had been implanted 24 h earlier interscapularly with a pellet of 17 β -estradiol (Innovative Research of America, Sarasota, FL). Once palpable tumors developed, their bi-directional dimensions in millimeters were measured using calipers, and tumor weights in milligrams were calculated using the formula for a prolate ellipsoid, $(L \times W^2)/2$, where *L* is the longer of the two dimensions. When tumors of 100-mg size developed, the mice were randomized to either a control diet (Lab Isopro RMH 3000; PMI Nutrition International, Inc., Brentwood, MO) or diets supplemented with curcumin. One day later, they received i.p. injections with filter-sterilized PBS or an equivalent volume of PBS containing cyclophosphamide. The impact on tumor weights was monitored for another 48 h with data expressed as a fold-change from day 0 for each animal. All measurements were performed by a technician who was blinded to the treatment assignments. The fold tumor weight differences between the curcumin diet and regular diet groups were compared for the fold differences from day 0 to day 2 and for the fold differences from day 1 to day 2 with the robust nonparametric Wilcoxon Two-Group test using normal scores.

Immunohistochemistry. Three mice from each treatment assignment in a separate group of animals were euthanized using guidelines of the American Veterinary Medical Association's Panel on Euthanasia. Tumors were then excised and fixed in Tissue-Tek O.C.T. (Sakura Finetek USA, Inc., Torrance, CA) while frozen in liquid nitrogen. For detection of apoptosis-associated ssDNA generation (35), sections were washed in 6:1 methanol:water, air dried, and treated with proteinase K (USB Corp., Cleveland, OH). The slides were then rinsed and heated at 56°C in prewarmed 50% formamide (USB Corp.), followed by rinsing in ice-cold PBS. After blocking, they were stained with the murine monoclonal antibody Mab 3299 (Chemicon International, Temecula, CA), with the secondary antibody consisting of cy3 fluorescent-conjugated goat antimouse antibody (Chemicon International). Sections were incubated for 15 min, washed in PBS, and mounted with a 4',6'-diamidino-2-phenylindole nuclear stain (Vector Laboratories, Burlingame, CA). Slides were visualized using an UV Zeiss Axioplan fluorescence microscope (Carl Zeiss Optical, Inc., Chester, VA). Separate photographs were taken with appropriate filters for blue nuclear staining and red ssDNA staining, overlaid using Adobe Photoshop software, and displayed as a fusion image at $\times 10$. The quantification of pixels was performed using NIH Image version 1.61 with uncalibrated absorbance settings and expressed as the mean \pm SE from five measurements in each section. Immunofluorescence for JNK was performed in a similar fashion using the murine monoclonal G9 antibody to phospho-SAPK/JNK (Cell Signaling Technology, Inc.).

RESULTS

Curcumin and Camptothecin-induced Apoptosis. To explore the possibility that curcumin could compromise the proapoptotic activity of chemotherapy, studies were pursued initially with MCF-7 human breast carcinoma cells and camptothecin. This topoisomerase 1 inhibitor is cytotoxic to breast cancer cells in animal tumor models using patient-derived cell lines (36), and generates ROS (37) and activates JNK during its induction of apoptosis (38). Mock-, vehicle-, and curcumin-treated cells demonstrated few, if any, low molecular weight apoptosis-associated DNA fragments and, whereas camptothecin induced large amounts of oligonucleosomal laddering, in the presence of curcumin, this appeared to be significantly blunted (Fig. 1A). A quantitative evaluation was then sought using an ELISA to detect histone-associated oligonucleosome DNA fragments. Compared with vehicle-treated cells, camptothecin induced a 5.40 ± 0.56 -fold increase in this apoptosis-associated parameter, but in the presence of curcumin, this increase was only 1.46 ± 0.12 -fold (Fig. 1B). Because curcumin has been reported to block DNA fragmentation without interfering with apoptosis in T lymphocytes (30), caspase 3 activity assays were performed as an independent assessment of apoptotic changes. Camptothecin induced a 2.69 ± 0.12 -fold increase in caspase 3 activity compared with the vehicle control, but with curcumin present this increase was only 1.53 ± 0.14 -fold (Fig. 1C). Curcumin itself was able to decrease basal caspase 3 activity in this assay to 0.65 ± 0.05 -fold of control levels, further demonstrating its ability to inhibit apoptosis-associated events in this cell line.

To better characterize the interaction between curcumin and camptothecin, studies were pursued with differing exposure times and concentrations. Inhibition of apoptosis was time dependent, but even a relatively brief 3-h incubation inhibited apoptosis by $18.2 \pm 2.6\%$ (Fig. 1D). Longer incubations of 6 and 9 h resulted in increasing inhibition of 36.9 ± 6.7 and $56.7 \pm 2.4\%$, respectively, after which a plateau was reached, during which additional exposure times did not significantly decrease programmed cell death. Curcumin was able to

inhibit camptothecin-induced apoptosis in a concentration-dependent fashion, with 1.0, 5.0, and 10.0 μM curcumin inhibiting apoptosis by 9.0 ± 4.7 , 43.9 ± 0.9 , and $66.3 \pm 1.6\%$, respectively (Table 1).

Curcumin and Alkylating Agent- and Anthracycline-induced Apoptosis. Alkylating agents and anthracyclines are commonly applied to the therapy of patients with breast cancer, and because both classes of drugs generate ROS (39–42) and activate JNK (43, 44), it was of interest to determine whether curcumin could inhibit their ability to induce apoptosis. MCF-7 cells were therefore exposed either to mechlorethamine or Adriamycin in culture, and the impact of curcumin on apoptosis was then evaluated. Mechlorethamine was used as the alkylating agent rather than cyclophosphamide because, although the latter is more frequently used clinically, it must be transformed *in vivo* to the active metabolite 4-hydroxycyclophosphamide to exert its effects (45). Curcumin was able to inhibit the ability of both mechlorethamine and Adriamycin to induce programmed cell death of MCF-7 cells in a concentration-dependent fashion (Table 1). With respect to Adriamycin, for example, curcumin at 1.0, 5.0, and 10.0 μM decreased induction of apoptosis by 47.2 ± 6.9 , 55.5 ± 4.4 , and $65.3 \pm 6.7\%$, respectively. To evaluate other model systems, MDA-MB-231 and BT-474 human breast cancer cell lines were used. In all of the cases studied, curcumin inhibited the ability of the chemotherapeutic drugs to activate apoptosis. This effect was dose dependent but even at 1.0 μM , a concentration documented in Phase I chemoprevention trials (25), curcumin significantly blocked apoptosis by up to 19.3 ± 2.2 or $27.5 \pm 16.7\%$, in the case of BT-474 cells treated with mechlorethamine or camptothecin, respectively.

Curcumin and Generation of ROS. ROS are important intermediates in the induction of apoptosis by chemotherapeutic drugs such as camptothecin and alkylating agents (33, 37, 46). Because curcumin acts as a free radical scavenger, it was of interest to evaluate its impact on generation of ROS using dichlorodihydrofluorescein diacetate. By itself, curcumin had a minimal impact on ROS in vehicle-treated MCF-7 cells, but treatment with camptothecin induced a 2.58-fold

Fig. 1. Curcumin inhibits camptothecin-induced apoptosis in MCF-7 cells. **A**, MCF-7 breast carcinoma cells were exposed for 12 h to either vehicle (Lane 3), 10 μM curcumin (Lane 4), 10 μM camptothecin (Lane 5), or both camptothecin and curcumin (Lane 6), and compared with mock-treated cells (Lane 2). Apoptosis-associated DNA fragmentation was evaluated by agarose gel electrophoresis with λ digested with *Hind*III (Lane 1) and a 100-bp ladder (Lane 7) as standards. **B**, after MCF-7 cells were treated with vehicle, curcumin, camptothecin, or curcumin and camptothecin, apoptosis was evaluated using the Cell Death Detection ELISA^{PLUS} kit. Results are the mean and SE from four experiments expressed as the fold increase in apoptosis compared with the vehicle control, arbitrarily set at 1.00. **C**, caspase 3 activity assays were performed on MCF-7 cells using the substrate acetyl-aspartyl-glutamyl-valyl-aspartate. Results are the mean \pm SE from three experiments, expressed as the ratio of absorbance of each sample to that of the vehicle control, arbitrarily set at 1.00. **D**, time dependence was studied by treating MCF-7 cells with 10.0 μM camptothecin for 12 h. Curcumin was either preincubated for 3 h and then present throughout camptothecin treatment for a total of 15 h, added at the same time as camptothecin, or at 3-h intervals thereafter. The percentage of inhibition of apoptosis was calculated as [(fold induction of apoptosis by camptothecin alone – fold induction in the presence of curcumin) / (fold induction of apoptosis by camptothecin alone) \times 100]. The mean percentage inhibition \pm SE is shown from four experiments.

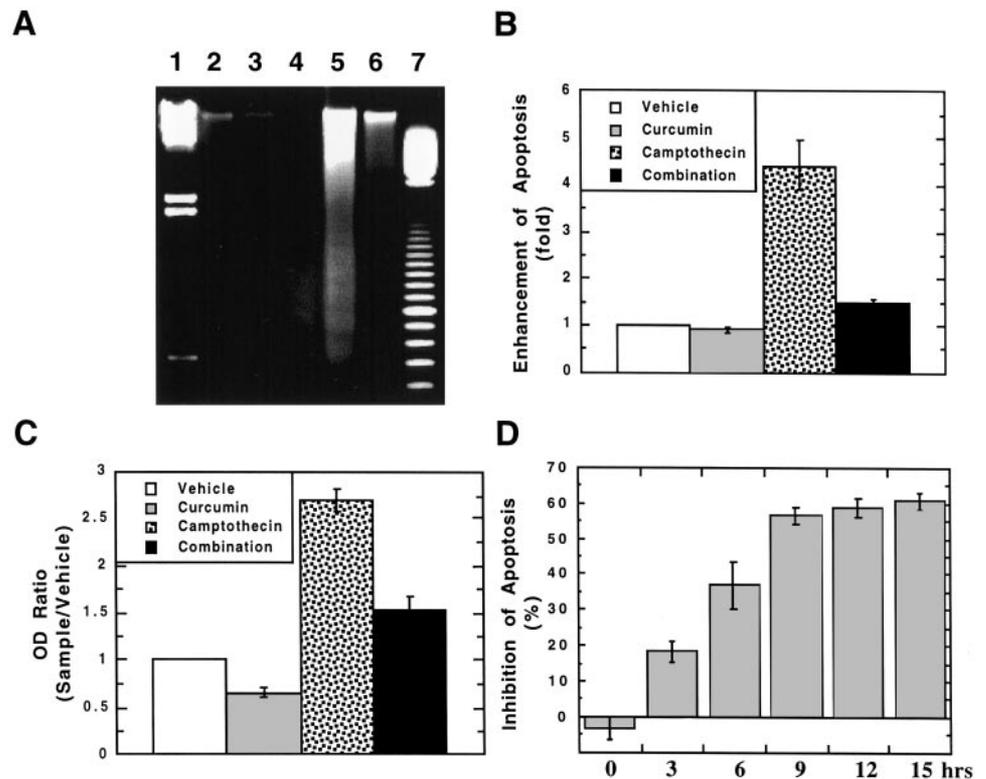


Table 1 Inhibition of chemotherapy-induced apoptosis (%)^a

Chemotherapeutic	Curcumin concentration	Breast cancer cell line		
		MCF-7	MDA-MB-231	BT-474
Camptothecin	1 μ M	9.0 \pm 4.7	0.3 \pm 9.6	27.5 \pm 16.7
	5 μ M	43.9 \pm 0.9	19.9 \pm 3.8	50.5 \pm 2.5
	10 μ M	66.3 \pm 1.6	43.2 \pm 4.4	71.2 \pm 10.5
Methlorethamine	1 μ M	2.6 \pm 3.3	5.3 \pm 6.0	19.3 \pm 2.2
	5 μ M	27.0 \pm 8.5	20.5 \pm 5.2	47.6 \pm 12.5
	10 μ M	23.3 \pm 1.0	22.7 \pm 5.4	70.4 \pm 12.3
Adriamycin	1 μ M	47.2 \pm 6.9	6.2 \pm 6.2	ND ^b
	5 μ M	55.5 \pm 4.4	18.0 \pm 2.9	ND
	10 μ M	65.3 \pm 6.7	38.5 \pm 4.6	ND

^a All results are the mean and SE from four experiments.

^b ND, not done.

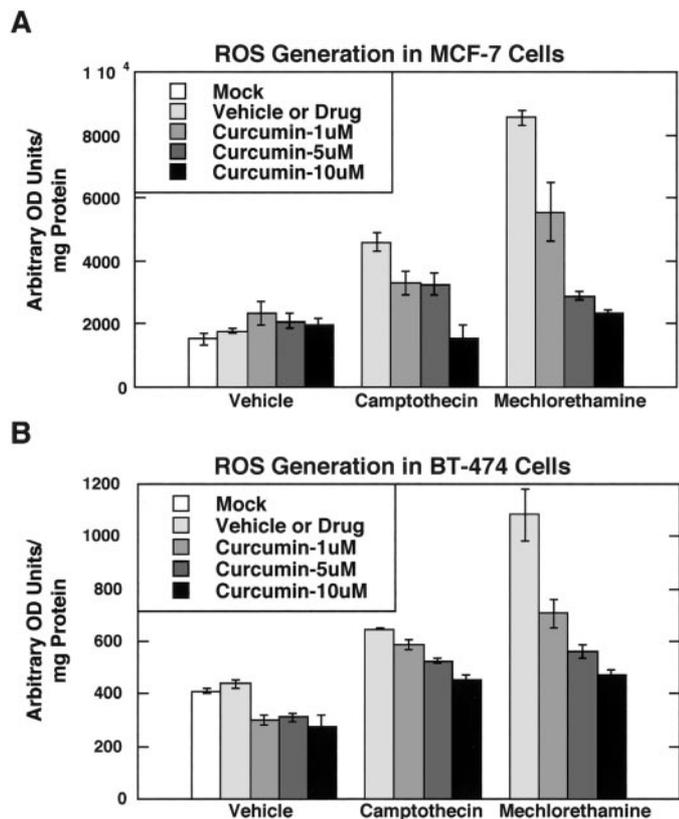


Fig. 2. Curcumin inhibits generation of ROS. MCF-7 (A) and BT-474 (B) cells were either mock treated, vehicle treated, or exposed to curcumin at 1, 5, and 10 μ M. In parallel, they were exposed to either 10 μ M camptothecin or 100 μ M mechlorethamine, with or without curcumin, for 12 h. The generation of ROS is assayed using dichlorodihydrofluorescein diacetate and is expressed as absorbance/mg of protein. The mean \pm SE is shown from three experiments, each performed in duplicate.

increase, whereas mechlorethamine induced a 4.80-fold increase in ROS (Fig. 2A). When curcumin was present in conjunction with either of these two chemotherapeutic agents, it inhibited ROS generation in a dose-dependent fashion. In the case of mechlorethamine, for example, 1, 5, and 10 μ M curcumin inhibited induction of ROS by 35.0, 66.1, and 72.6%, respectively, compared with the alkylating agent alone. A similar trend was noted in BT-474 cells in which curcumin inhibited ROS by 34.7, 48.1, and 56.6%, respectively, (Fig. 2B) at the concentrations tested. Indeed, 10 μ M curcumin decreased ROS in camptothecin- and mechlorethamine-treated MCF-7 and BT-474 cells to levels comparable with vehicle-treated cells, demonstrating curcumin's ability to act as a free radical scavenger.

Curcumin and JNK Activation. ROS have been reported to stimulate SAPK pathways such as JNK (47, 48), and because curcumin

inhibits JNK and AP-1 signaling (16, 17), we sought to determine its impact on chemotherapy-induced JNK activation. Using an *in vitro* immunocomplex assay, curcumin appeared to have the ability to inhibit JNK activation in a dose-dependent fashion in MCF-7 cells treated with camptothecin (data not shown). To characterize this further, AP-1 activity was probed using an ELISA that detects phospho-c-Jun levels. By itself, curcumin had a mild impact on AP-1 in vehicle-treated MCF-7 cells, but treatment with mechlorethamine induced a 1.29-fold increase, whereas camptothecin induced a 2.63-fold increase in AP-1 activity (Fig. 3A). When curcumin was present with either of these two agents, it inhibited AP-1 activation, and in most cases did so in a dose-dependent fashion. In the case of camptothecin, for example, 1, 5, and 10 μ M curcumin inhibited AP-1 activation by 35.0, 39.5, and 44.9%, respectively, compared with camptothecin alone. A similar trend was noted in camptothecin-treated BT-474 cells in which curcumin inhibited AP-1 activation by 15.4, 27.7, and 63.7%, respectively (Fig. 3B), at the concentrations tested.

Curcumin and Mitochondrial Cytochrome *c* Release. Because ROS (reviewed in Ref. 49) and JNK activation (50) impact cytochrome *c* release from mitochondria, which then activates apoptosis (reviewed in 51), we postulated that curcumin might be decreasing mitochondrial cytochrome *c* release. Thus, the cytosolic fractions from cells were isolated, and the presence of cytochrome *c* was determined by Western blotting. Both camptothecin and mechlorethamine induced large amounts of cytochrome *c* release into the cytosol of MCF-7 cells, compared with vehicle-treated controls (Fig. 4A and B, respectively). However, curcumin was able, in a dose-dependent fashion, to decrease the levels of cytosolic cytochrome *c*. In the case

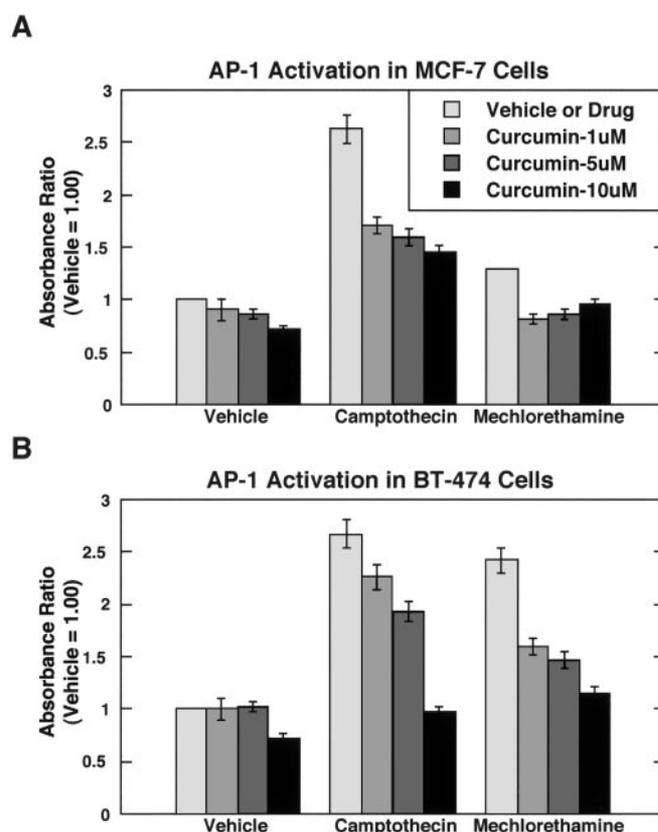


Fig. 3. Curcumin inhibits JNK activation. MCF-7 (A) and BT-474 (B) cells were treated as described in the legend to Fig. 2, and JNK/AP-1 activity was evaluated using an ELISA that detects phosphorylated c-Jun. The mean \pm SE is shown from two experiments, each performed in duplicate, with results expressed in relation to vehicle-only controls, which were arbitrarily set at 1.00.

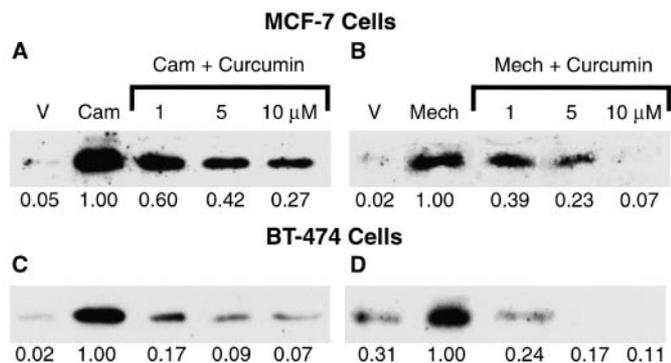


Fig. 4. Curcumin inhibits release of cytochrome *c* into the cytosol. MCF-7 cells were treated with vehicle and camptothecin (A) or mechlorethamine (B) either alone or in the presence of curcumin. The content of cytochrome *c* in the cytosol was evaluated by Western blotting, and equal loading was confirmed by reprobing each blot for heat shock cognate protein 70. Densitometry of the autoradiographs was performed for cytochrome *c* levels, corrected for loading, and the data are shown below each panel in relation to the chemotherapy agent alone, which was arbitrarily set at 1.00. BT-474 cells were similarly treated in C and D, respectively, and each panel is a representative result from one of two experiments. V, vehicle; Cam, camptothecin; Mech, mechlorethamine.

of camptothecin, for example, 1, 5, and 10 μ M curcumin decreased cytosolic cytochrome *c* levels by 40, 58, and 73%, respectively (Fig. 4A), whereas in camptothecin-treated BT-474 cells, curcumin decreased cytosolic cytochrome *c* by 83, 91, and 93%, respectively (Fig. 4C). These studies support the hypothesis that curcumin inhibits topoisomerase 1 inhibitor- and alkylating agent-mediated apoptosis by inhibiting ROS generation, JNK activation, and mitochondrial cytochrome *c* release.

Curcumin and Breast Cancer Xenografts after Cyclophosphamide. The ability of curcumin to inhibit chemotherapy-mediated apoptosis in culture suggested that it might have the same activity *in vivo*. To evaluate this possibility, xenograft models of human breast cancer were prepared in nude (*nu/nu*) mice, and these were randomized to either a standard diet or one supplemented with curcumin at 25 g/kg of feed. This represents 8.7 mm/kg of body weight/day (1, 2, and 4). Twenty-four h later, these mice were treated with a single injection of cyclophosphamide, an alkylating agent that is a component of several regimens used to treat patients with breast cancer, and the impact on their tumors was followed daily for 2 days. In pilot experiments with both MCF-7-based and BT-474-based xenografts, the animals receiving a regular diet treated with cyclophosphamide had a significant decrease in tumor size from the day of treatment, or day 1, to day 2. The animals receiving a curcumin-supplemented diet treated with cyclophosphamide, however, did not have a significant decrease in tumor size over this time period. Power calculations were then performed to determine the sample size needed to obtain statistically significant results, and a second study was performed using the BT-474 model system. Animals treated with cyclophosphamide feeding on a standard diet had a significant decrease in their tumor size from day 1 to day 2, after which the tumors began growing once again (Fig. 5). When animals feeding on a curcumin-supplemented diet were treated with cyclophosphamide, however, their tumors did not decrease in size and continued to grow from day 1 to day 2 and then to day 3 (Fig. 5). Indeed, whereas tumors in the standard diet group had increased by only 1.10 ± 0.18 -fold from day 0 to day 2, showing the effectiveness of day 1 cyclophosphamide therapy, the tumors in the curcumin diet group had increased by 2.58 ± 0.24 -fold compared with day 0 despite cyclophosphamide treatment ($P < 0.0001$). In contrast, control experiments showed no such differences in vehicle-treated animals (data not shown), with tumors in the standard diet group increasing by 2.49 ± 0.36 -fold from day 0 to day 2, whereas

tumors in the curcumin-supplemented diet increased by 1.97 ± 0.18 -fold.

Apoptosis and JNK Activation in Xenograft Tissue. To determine whether the decreased effectiveness of cyclophosphamide was attributable to inhibition of programmed cell death, tumor sections were prepared 24 h after cyclophosphamide. Apoptosis was then evaluated by probing for single-stranded sequences after formamide-induced DNA denaturation (35), and densitometry was performed to obtain quantitative results. Comparing the two cyclophosphamide-treated groups, there was 1.6-fold more apoptosis detected in the standard diet cohort (Fig. 6A) than the curcumin-diet cohort (Fig. 6B). Also, whereas cyclophosphamide increased apoptosis in the standard diet group compared with treatment with vehicle alone, in the curcumin diet group it did not increase the abundance of ssDNA (data not shown). The activity of the JNK signal transduction pathway was then evaluated using an antibody that recognizes phosphorylated, activated SAPK/JNK. Treatment of the standard diet group with cyclophosphamide resulted in a significant increase in phospho-JNK reactivity (Fig. 6C), compared with treatment of the curcumin diet group (Fig. 6D). Indeed, this latter group showed no increased fluorescence density compared with that of the vehicle-treated curcumin diet group or the vehicle-treated standard diet group. These studies support the hypothesis that *in vivo*, as it does in tissue culture, curcumin can inhibit alkylating agent-induced apoptosis and JNK activation.

DISCUSSION

The chemopreventive activity of curcumin in several animal tumor model systems has led investigators to examine its potential impact on apoptosis with varying results. Some reports suggest that curcumin inhibits apoptosis such as in human and rat T lymphocytes (30), whereas others have documented an induction of apoptosis in lines such as HL60 cells (29) and *in vivo* in azoxymethane-induced colon tumors (52). In some cell lines, there have even been conflicting results such as in HT-29 human colon cancer cells that have been noted to be induced into apoptosis by some (53), whereas others have noted no effect of curcumin (27). With respect to cytotoxic chemotherapy, curcumin has been reported to protect rat lungs and myocardium from injury by bleomycin (31) and Adriamycin (32), respectively, and inhibit apoptosis in UV-irradiated Jurkat cells and

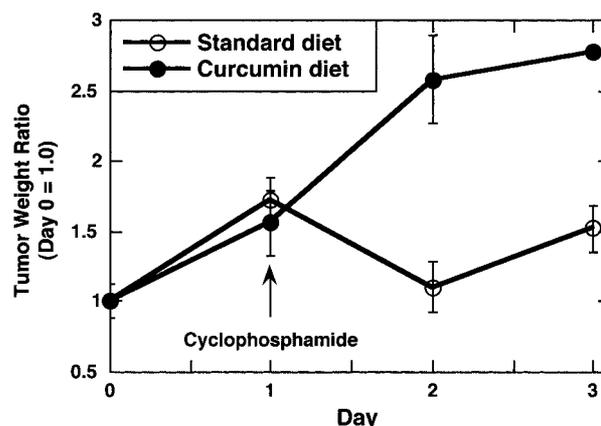
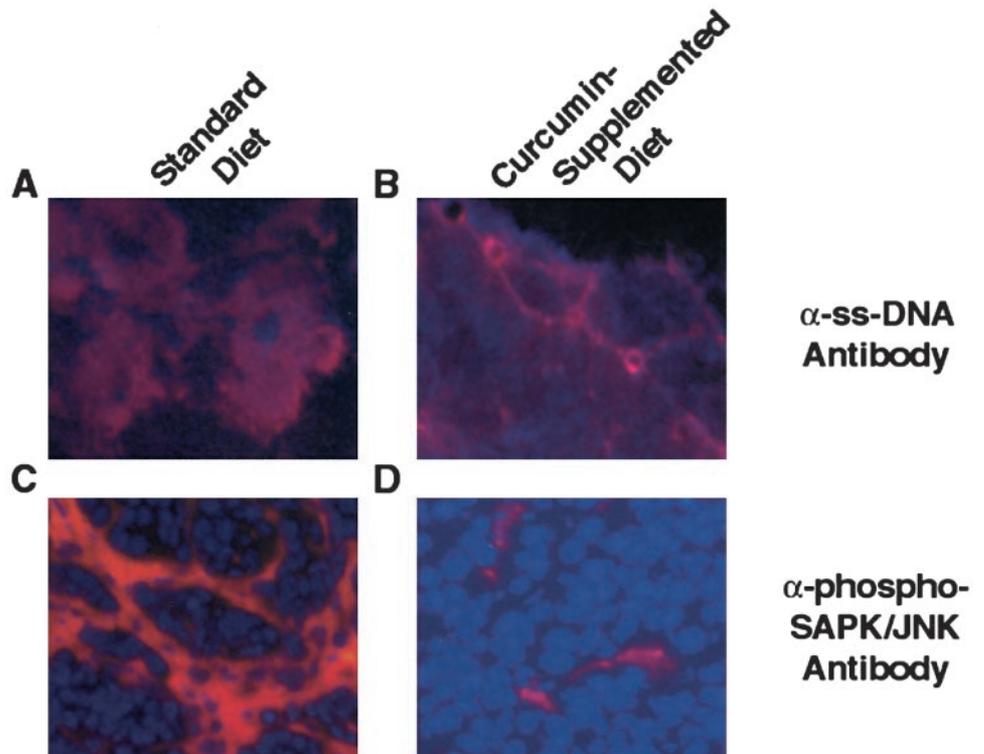


Fig. 5. Dietary curcumin blunts cyclophosphamide-mediated tumor growth inhibition. Nude mice bearing BT-474-based xenografts were randomized on day 0 to receive either standard or curcumin-supplemented diets and on day 1 were treated with a single i.p. dose of cyclophosphamide at 265 mg/kg. Tumor weights were followed for a total of 3 days, calculated using the tumor dimensions, and plotted as the ratio of the tumor weight to that on day 0, which is arbitrarily set at 1.00. Sixteen mice were included in each of these treatment groups, whereas two additional groups were treated with either a standard diet and vehicle or the curcumin-supplemented diet and vehicle. These results are described in the text.

Fig. 6. Dietary curcumin inhibits apoptosis and JNK activation *in vivo*. One day after treatment with cyclophosphamide, BT-474-based tumor xenografts were excised, sectioned, and prepared for immunofluorescence. Apoptosis induced by cyclophosphamide is shown in the standard diet group (A) and in the curcumin-supplemented diet group (B) as red areas containing ssDNA. Phospho-JNK staining is shown in C and D for these two groups, respectively, with red areas indicating presence of phosphorylated, activated JNK. The background blue staining in all panels is a 4',6-diamidino-2-phenylindole nuclear stain. Control slides from vehicle-treated standard diet and curcumin diet groups were prepared as well. The density of immunofluorescence staining discussed in the text is the mean from five separate fields on two duplicate slides.



dexamethasone-treated thymocytes (54), but it could not inhibit etoposide-induced apoptosis in U937 human monoblastic leukemia cells (55). Many of these investigations have used extended incubation periods with very high curcumin concentrations, however, that would be unlikely to be achieved as a result of dietary curcumin intake by cancer patients. Therefore, we sought to study the effects of curcumin using conditions that more closely reproduced those that could be found *in vivo*. Because of its ability to act as a free radical scavenger (6–8) and to inhibit AP-1 (16) and JNK activation (17), we hypothesized that because these are important steps in the ability of some cytotoxic DNA-damaging drugs to induce apoptosis, dietary curcumin might inhibit the effectiveness of such cancer chemotherapy.

In this study, we demonstrate that curcumin was able, in a dose- and time-dependent fashion, to inhibit camptothecin-mediated apoptosis in MCF-7 breast cancer cells (Fig. 1). This occurred at concentrations that have been documented in a Phase I chemoprevention trial in humans, where serum curcumin levels ranging from 0.51 ± 0.11 to $1.77 \pm 1.87 \mu\text{M}$ were noted (25). Serum levels in humans after an oral dose of curcumin peak rapidly in as little as 1–2 h, but they decline much more slowly over the next 12 h (25), and in our studies, even a brief 3-h exposure to curcumin was sufficient to significantly inhibit apoptosis. Moreover, this inhibition was demonstrable in a concentration-dependent manner using the topoisomerase I inhibitor camptothecin, the alkylating agent mechlorethamine, and the anthracycline Adriamycin, not only in MCF-7 cells but also in MDA-MB-231 and BT-474 human breast cancer cells. Such findings suggest that this activity is independent of p53 status because MCF-7 cells are p53 wild type, whereas MDA-MB-231 and BT-474 cells have mutant p53. Hormone receptor status would also appear to not be a significant influence because MCF-7 cells are estrogen receptor positive, whereas MDA-MB-231 cells are receptor negative (56). However, additional experiments, *e.g.*, comparing parental MDA-MB-231 cells and MDA-MB-231 clones that express wild type p53, will be necessary to test these hypotheses directly.

To evaluate possible mechanisms responsible for this inhibition of

apoptosis, we studied ROS generation and found that curcumin could, in a dose-dependent fashion, inhibit the camptothecin- and mechlorethamine-induced production of ROS (Fig. 2). JNK activation with AP-1 activity (Fig. 3) and mitochondrial release of cytochrome *c* (Fig. 4) was also inhibited in a concentration-dependent manner. These findings support the hypothesis that curcumin inhibits apoptosis by blocking ROS formation and JNK activation, both of which are important signals for cytochrome *c* release from mitochondria into the cytoplasm, which triggers caspase-mediated programmed cell death. If confirmed, such a mechanism would allow the prediction that drugs that do not activate JNK should not be influenced by curcumin. Consistent with this assumption, methotrexate and 5-fluorouracil, two drugs used in the care of breast cancer patients, which function as antimetabolites and are not known to activate JNK, were able to induce apoptosis without any impact by curcumin (data not shown). It should be noted, however, that Bhaumik *et al.* (57) have reported that in AK-5 rat histiocytoma cells, curcumin alone was able to induce apoptosis through ROS production and cytochrome *c* release. This difference may be attributable to the experimental conditions because Bhaumik *et al.* used a concentration of $50 \mu\text{M}$ curcumin and did not study its impact in the presence of a chemotherapeutic agent. Alternatively, this may indicate that there is some cell type specificity to the impact of curcumin. The current studies also do not rule out a possible involvement of other pathways that are impacted upon by curcumin in blocking apoptosis. For example, activation of the transcription factor NF- κ B is important in paclitaxel-induced apoptosis (58), and because curcumin inhibits NF- κ B (14, 15), this may be another pathway involved in its antiapoptotic activity. Additional ongoing studies using dominant negative mutant constructs that will selectively inactivate only one pathway at a time will hopefully prove instructive in elucidating the molecular basis for this function of curcumin.

Given the ability of curcumin to inhibit apoptosis in tissue culture, we sought to determine whether it could also do so *in vivo*, and we found that dietary supplementation significantly decreased cyclophos-

phamide-induced tumor growth delay (Fig. 5). Immunofluorescence studies indicated that this occurred in conjunction with decreased apoptosis and also with decreased JNK activation (Fig. 6). This result was somewhat surprising in that, whereas serum curcumin concentrations in chemoprevention trials have been comparable with those used in our *in vitro* studies, these occurred at a daily curcumin dose of 8000 mg (25). Even in populations where dietary curcumin intake is high, daily exposure is only on the order of 200 mg (1–4). Therefore, it might seem unlikely that such diets would result in systemic curcumin levels approaching the conditions we used *in vitro*, especially since the oral bioavailability of curcumin is low because it is extensively metabolized by the liver. Studies of tumor sections, however, revealed that those from the two curcumin-supplemented diet groups showed an immunofluorescence characteristic of curcumin, whereas the standard diet groups did not (data not shown; Refs. 1–3). This supports the possibility that, even after only short-term feeding, curcumin, or one of its metabolites, can be present in tumor tissue. One possible explanation for the ability of dietary curcumin to inhibit apoptosis at low levels of exposure *in vivo* is that this may represent a difference in the absorption and metabolism of curcumin between humans and animals. A recent study comparing human and rat hepatocytes, however, found that the major metabolites in both were hexahydrocurcumin and hexahydrocurcuminol (59). Alternatively, although curcumin metabolites have been shown to be less able to inhibit phorbol ester-induced prostaglandin E₂ production in human colonic epithelial cells than the parental compound (59), they may be more active than curcumin in their ability to inhibit ROS formation, JNK activity, and cytochrome *c* release. Finally, it is possible that some of the antiapoptotic effect of curcumin *in vivo* does not require it to be present at the site of the tumor. Cyclophosphamide is a prodrug activated by cytochrome P450s in the liver (45), and curcumin inhibits several P450 isoenzymes (18). The higher concentrations of curcumin one would expect to find in the liver after a meal containing this agent may be sufficient to inhibit conversion of cyclophosphamide to its active metabolites, thereby limiting its antitumor efficacy.

Curcumin's ability to inhibit apoptosis in our *in vivo* studies suggests that patients with breast cancer who are receiving active cytotoxic chemotherapy should be excluded from enrollment in any curcumin-based chemoprevention trials in the future. Because curcumin has antiangiogenic properties and may have therapeutic potential in human prostate cancer (24), our findings also suggest care in its application to patients with other malignancies receiving concurrent chemotherapy until this activity of curcumin can be further investigated. In addition, curcumin is lipophilic and up to 80% is absorbed by passive diffusion from the intestinal lumen, after which it concentrates in membranous cellular structures (54). It is possible, therefore, that the effects of curcumin might be amplified in patients with colon cancer receiving chemotherapy unless, as noted above, it has some cell type specificity, or its antiapoptotic effects are mediated through hepatic cytochrome P450. Finally, these findings strongly support additional research to determine whether breast cancer, and possibly other cancer patients undergoing chemotherapy, should limit dietary supplementation of curcumin, which is widely available over the counter as turmeric extracts, and possibly even avoid curcumin-containing foods altogether.

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Dietary Curcumin Inhibits Chemotherapy-induced Apoptosis in Models of Human Breast Cancer

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