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Antiproliferative effect of curcumin (diferuloylmethane) against human breast tumor cell lines

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Pharmacologically safe compounds that can inhibit the proliferation of tumor cells have potential as anticancer agents. Curcumin, a diferuloylmethane, is a major active component of the food flavor turmeric (*Curcuma longa*) that exhibits anticarcinogenic properties *in vivo*. *In vitro*, it suppressed c-*jun*/Ap-1 and NF-κB activation and type 1 human immunodeficiency virus long-terminal repeat-directed gene expression. We examined the antiproliferative effects of curcumin against several breast tumor cell lines, including hormone-dependent and -independent and multidrug-resistant (MDR) lines. Cell growth inhibition was monitored by [^{3}H]thymidine incorporation, Trypan blue exclusion, crystal violet dye uptake and flow cytometry. All the cell lines tested, including the MDR-positive ones, were highly sensitive to curcumin. The growth inhibitory effect of curcumin was time- and dose-dependent, and correlated with its inhibition of ornithine decarboxylase activity. Curcumin preferentially arrested cells in the G_2/S phase of the cell cycle. Curcumin-induced cell death was neither due to apoptosis nor to any significant change in the expression of apoptosis-related genes, including Bcl-2, p53, cyclin B and transglutaminase. Overall our results suggest that curcumin is a potent antiproliferative agent for breast tumor cells and may have potential as an anticancer agent.

Key words: Cell cycle, breast carcinoma, drug resistance, flavonoids, ornithine deoxycarboxylase.

Introduction

Curcumin (diferuloylmethane), a non-nutritive food chemical present in turmeric (*Curcuma longa*), inhibits lipid-peroxide-induced DNA damage and tumor initiation induced by benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene.[1–4] Phorbol ester-induced tumor promotion is also inhibited by curcumin.[5,6] Besides its anticarcinogenic effects, curcumin exhibits anti-inflammatory properties *in vivo*.[7–9] Pharmacological safety of curcumin is well established due to the fact that people in certain countries have for centuries consumed curcumin as a dietary spice in amounts up to 100 mg/day without any harm.[7]

In vitro curcumin inhibits neutrophil activation, suppresses mitogen-induced proliferation of blood mononuclear cells, inhibits the mixed lymphocyte reaction and inhibits proliferation of smooth muscle cells.[10,11] It is also a potent scavenger of reactive oxygen species, protects hemoglobin from nitrite-induced oxidation to methemoglobin and inhibits lipid peroxidation.[12–14] Some of these activities are also responsible for its ability to protect DNA from free radical-induced damage and hepatocytes from damage by various toxins.[14,15]

How curcumin induces such a wide range of effects is not fully understood. Among the possibilities are its profound ability to inhibit the phorbol ester-induced expression of the nuclear transcription factor c-*jun*/AP-1,[16] inhibition the nuclear transcription factor NF-κB,[17] and inhibition of protein kinase C (PKC) and xanthine dehydrogenase/oxidase.[18,19] Ornithine decarboxylase (ODC; EC 4.1.1.17), a rate-limiting enzyme in polyamine synthesis, and tyrosine protein kinase are inhibited by curcumin.[20] It also inhibits the activity of several different serine/threonine and tyrosine kinases *in vitro*.[21] More recently, curcumin was shown to inhibit both the epidermal growth factor (EGF) receptor intrinsic kinase activity and EGF-mediated activation of EGF receptor phosphorylation.[22,23] These studies suggest that curcumin may serve as a growth inhibitory agent by interfering with certain signal transduction pathways that are critical for cell growth.

The potential of curcumin as an anticancer agent led us to investigate the effect of this compound on proliferation and cell growth of several breast tumor cells. The results described in this paper suggest that curcumin could serve as an effective antitumor agent

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against both hormone-dependent and -independent human breast cancer cells even when they become refractory to conventional chemotherapy.

Materials and methods

Materials

Highly purified curcumin was purchased from Sigma (St Louis, MO). Dulbecco's modified Eagle's medium (DMEM/F12) was obtained from ICN Biomedical (Irvine, CA); RPMI 1640, DMEM and EMEM from Whittaker MA Bioproducts (Walkersville, MD); fetal bovine serum (FBS) and gentamicin from Gibco (Grand Island, NY). Tetrachloro-diphenylglycouril, glycine and 3-(4-5-dimethylthiozol-2-yl)2-5-diphenyl-tetrazolium bromide (MTT) were obtained from Sigma. The antibodies to human Bcl-2, p53 and guinea-pig liver tissue type transglutaminase (TGase) were purchased from Neo-Markers (Fremont, CA) and anti-cyclin B from Santa Cruz Biotechnology (Santa Cruz, CA).

Cells

The human breast tumor cell lines BT-20, T-47D, SK-BR3 and MCF-7 were obtained from the ATCC (Rockville, MD). The MCF-7 cells were selected for resistance to adriamycin ($MCF\text{-}7_{ADR}$) and BT-20 cells were selected for resistance to tumor necrosis factor ($BT\text{-}20_{TNF}$) as previously described.[24] Cells were tested for Mycoplasma contamination using either the DNA-based assay kit purchased from Gen-Probe (San Diego, CA) or the Hoechst stain.

Cell culture

All breast tumor cell lines were routinely grown in RPMI 1640 medium supplemented with 10 mM HEPES buffer, 2 mM glutamine, 50 μg/ml gentamicin and 10% FCS. The cells were cultured in a humidified incubator in 5% CO_2 in air and were maintained in continuous exponential growth by twice a week passage.

Cell proliferation assays

Cell growth assays were carried out essentially according to the procedure described.[25] Briefly, the cells (5×10^3/well) were plated in 0.2 ml of the medium (RPMI 1640 with 10% FBS) in 96-well Corning plates in the presence or absence of indicated concentrations of the drug. At appropriate times, the medium was removed and cells were either counted by a hemocytometer or monitored by crystal violet staining. In preliminary experiments we determined that the crystal violet staining method to determine cell viability correlates well with cell number determined by detachment with a trypsin solution and counting with a hemocytometer. Relative cell viability was calculated by dividing optical density in the presence of the test sample by optical density in the absence of the test sample (medium) and multiplying the results by 100.

For [^{3}H]thymidine incorporation, cells were cultured and treated with curcumin as indicated above. During the last 6 h, [^{3}H]thymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA) was added to each well (0.5 μCi/well). Thereafter, the culture medium was removed, and the cells washed twice with phosphate-buffered saline (PBS) and detached in a 0.5% trypsin solution containing 5.3 mM EDTA. The cell suspension was then harvested with the aid of a Filtermate 196 cell harvester (Packard Instruments, Canberra, Australia) and lysed by washing with distilled water. Radioactivity bound to the filter was measured directly by a Direct Beta Counter Matrix 9600 (Model 1600 TR; Packard, Meriden, CT). Thymidine incorporation determined by this method has been shown to correlate with cell growth.[26] All determinations were made in triplicate.

MTT assay

The number of viable cells remaining after appropriate treatment was determined by using the modified tetrazolium salt (MTT) assay as described.[24] Briefly, 5×10^3 cells/well were incubated in the presence or absence of the indicated test sample in a final volume of 0.2 ml for 72 h at 37°C. Thereafter, 0.1 ml of cell medium was removed and 0.025 ml of MTT solution (5 mg/ml in PBS) was added to each well. After 2 h incubation at 37°C, 0.1 ml of the extraction buffer (20% sodium dodecyl sulfate, 50% dimethyl formamide) was added. After an overnight incubation at 37°C, the optical densities at 570 nm were measured using a 96-well multiscanner autoreader (Dynatech MR 5000), with the extraction buffer serving as a blank. The cell viability was expressed as a percentage using the following equation:

$$\frac{A_{570} \text{ of treated sample}}{A_{570} \text{ of untreated sample}} \times 100$$

ODC assay

ODC activity in curcumin-treated breast tumor cells was determined according to a method described by Grewal *et al.*[27] Briefly, after appropriate treatment, the cells (8×10^6) were lysed by sonication in 25 mM Tris–HCl buffer containing 2.5 mM DTT and 0.1 mM EDTA. The reaction was carried out in 15 ml Falcon tubes containing 150 mM Tris-HCl (pH 7.5), 1 mM EDTA, 22.5 mM DTT, 0.4 mM pyridoxal phosphate and 4 mM L-[^{14}C]ornithine (New England Nuclear, Boston, MA) in a 0.2 ml volume. The reaction was initiated by adding 0.05 ml of the cell lysate (1 mg protein). The tubes were immediately capped and incubated at 37°C in a shaking water bath for 1 h. The cap contained a Whatman 3 mm filter disc saturated with barium hydroxide to trap $^{14}CO_2$ produced during the reaction. The reaction was stopped by adding 0.5 ml of 5 N HCl and incubtaion continued for an additional 1 h to ensure complete entrapment of the CO_2. The filter discs were recovered, dried and counted on a β scintillation counter. A parallel reaction with heat-inactivated (100°C for 10 min) cell lysate served as a control for background counts. A unit of enzyme activity represented the amount of enzyme that catalyzes 1 nmol of $^{14}CO_2$ released from ornithine/h/mg protein.

Cell cycle analysis

Curcumin-induced changes in the cell cycle of tumor cells were determined by using an Epics-Elite Laster flow cytometer (Coulter, Hialeah, FL). Briefly, trypsin-detached cells were washed in PBS and fixed in absolute methanol. About 1×10^6 alcohol-fixed cells were then washed in PBS and resuspended in 1 ml of lysis-staining solution that contained propidium iodide and a detergent (Coulter). The stained cells were analyzed for relative DNA content. Chicken erythrocytes (Coutler) were used as a standard. The number of cells in various phases of the cell cycle was determined with the aid of the Multicycle program (Phoenix Flow Systems, San Diego, CA). All experiments were repeated at least twice. Most of the data shown are averages of duplicate or triplicate determinations with below 10% variations.

Western blots

Following treatment with curcumin under appropriate conditions, cells were lysed in Tris–HCl buffer (20 mM, pH 7.2) containing 1 mM EDTA, 1 mM EGTA, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.1% aprotinin, 0.1% leupeptin, 0.1% pepstatin and 0.1% Triton X-100. The lystes were centrifuged at 16000 *g* for 45 min, and the clear supernatants were assayed for protein using the amido black dye staining procedure and bovine serum albumin as standard. Fifty micrograms of protein from each sample was electrophoresed on 10% SDS–polyacrylamide gel and subsequently electrotransferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat milk and probed with anti-Bcl-2, anti-p53, anti-TGase or anti-cyclin B1 antibody. The immunoreactive bands were detected by using horseradish peroxidase-conjugated anti-mouse IgG as secondary antibody and enhanced chemiluminescence detection system according to the manufacturer's instructions (Amersham, Arlington Heights, IL). The membranes were stripped, using a procedure recommended by the manufacturer (Amersham), and reprobed with antibody to actin (Santa Cruz Biotechnology).

Results

Curcumin inhibits the proliferation of different breast tumor cell lines

We first investigated the effect of curcumin on growth of different breast tumor cell lines by the thymidine incoporation assay. The results of these experiments are shown Table 1. Treatment of cells with 1 μg/ml (2.7 μM) curcumin for 72 h inhibited the growth of all the seven breast tumor cell lines tested with a maximum effect on BT-20 cells. Interestingly, the cell line (MCF-7_{ADR}) that exhibits

Table 1. Antiproliferative effect of curcumin against breast tumor cell lines

Cell lines	Relative cell viability (% of control)
BT-20	1 ± 0
BT-20_{TNF}	8 ± 0
SK-BR3	6 ± 1
MCF-7	9 ± 1
MCF-7_{ADR}	15 ± 6
T-47 D	13 ± 0
ZR-75-1	26 ± 1

Cells (5×10^3) were plated overnight and then incubated with curcumin (1 μg/ml). After 66 h at 37°C, the cells were pulsed with [^{3}H]thymidine for 6 h and then harvested. Thymidine incorporation was determined and normalized against untreated cells (100%). All determinations were made in triplicate.

an 80- to 100-fold increase in resistance to adriamycin was also senstive to curcumin-induced inhibition of cell growth. Similarly, a TNF-resistant subclone of BT-20 cells ($BT\text{-}20_{TNF}$) demonstrated significant growth inhibition when cultured in the presence of curcumin.

Curcumin inhibits growth of hormone-independent and -dependent breast tumor cells

The MCF-7 and T-47D cell lines are estrogen-dependent, whereas the SK-BR3 line is estrogen-independent. The growth of the estrogen-dependent cell lines was inhibited in a dose-dependent manner with maximum inhibition observed at less than a 1 μg/ml concentration of curcumin (Figure 1A and B). A similar dose-dependent inhibition was observed when the estrogen-independent SK-BR3 cells were incubated in the presence of curcumin (Figure 1C).

The effect of curcumin against hormone-dependent MCF-7 and hormone-independent BT-20 and MDA231 cell lines was time-dependent (Figure 2). A significant growth inhibitory effect of curcumin on thymidine incorporation by MCF-7 and BT-20 cells could be observed as early as 20 h after treatment (Figure 2A and 2B). When examined for the growth rate, 1 μg/ml of curcumin was sufficient to completely inhibit the growth of MCF-7 cells for up to 7 days (Figure 2C), whereas MDA231 cells required a high concentration of curcumin (3 μg/ml) to show complete inhibition of cell growth (Figure 2D). The drug-induced inhibition in thymidine incorporation was due to a net decrese in the number of viable cells remaining after curcumin treatment.

When compared to untreated cells, MCF-7 cells treated with curcumin for 24 h did not show any obvious changes in their morphology. However, 48 h treatment with curcumin in general, and 72 h treatment in particular, induced some characteristics morphological changes in MCF-7 cells (Figure 3). The treated cells typically appeared enlarged, occasionally with two to four nuclei, and showed significant changes in nuclear morphology (Figure 3B).

Next we determined the effect of curcumin on the ability of MCF-7 cells to form anchorage-dependent colonies. Ten thousand cells were seeded per

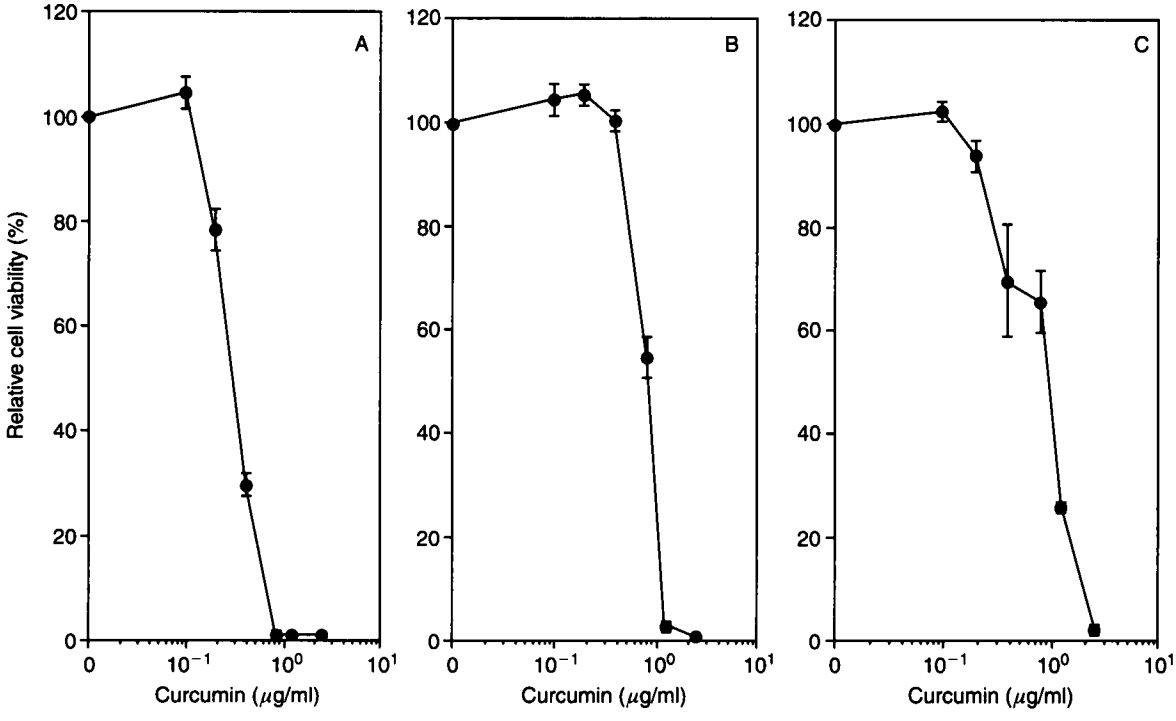

Figure 1. Dose-dependent growth inhibition of hormone-dependent MCF-7 (A) and T-47D (B), and of hormone-independent SK-BR3 (C) human breast adenocarcinoma cells. Cells (5×10^3 cells/0.1 ml/well) were plated overnight at 37°C, washed and then incubated with different concentrations of curcumin for 72 h. Viable cells were counted as described in Materials and methods. All determinations were made in triplicate. The results are expressed as percentages of the control (untreated cells).

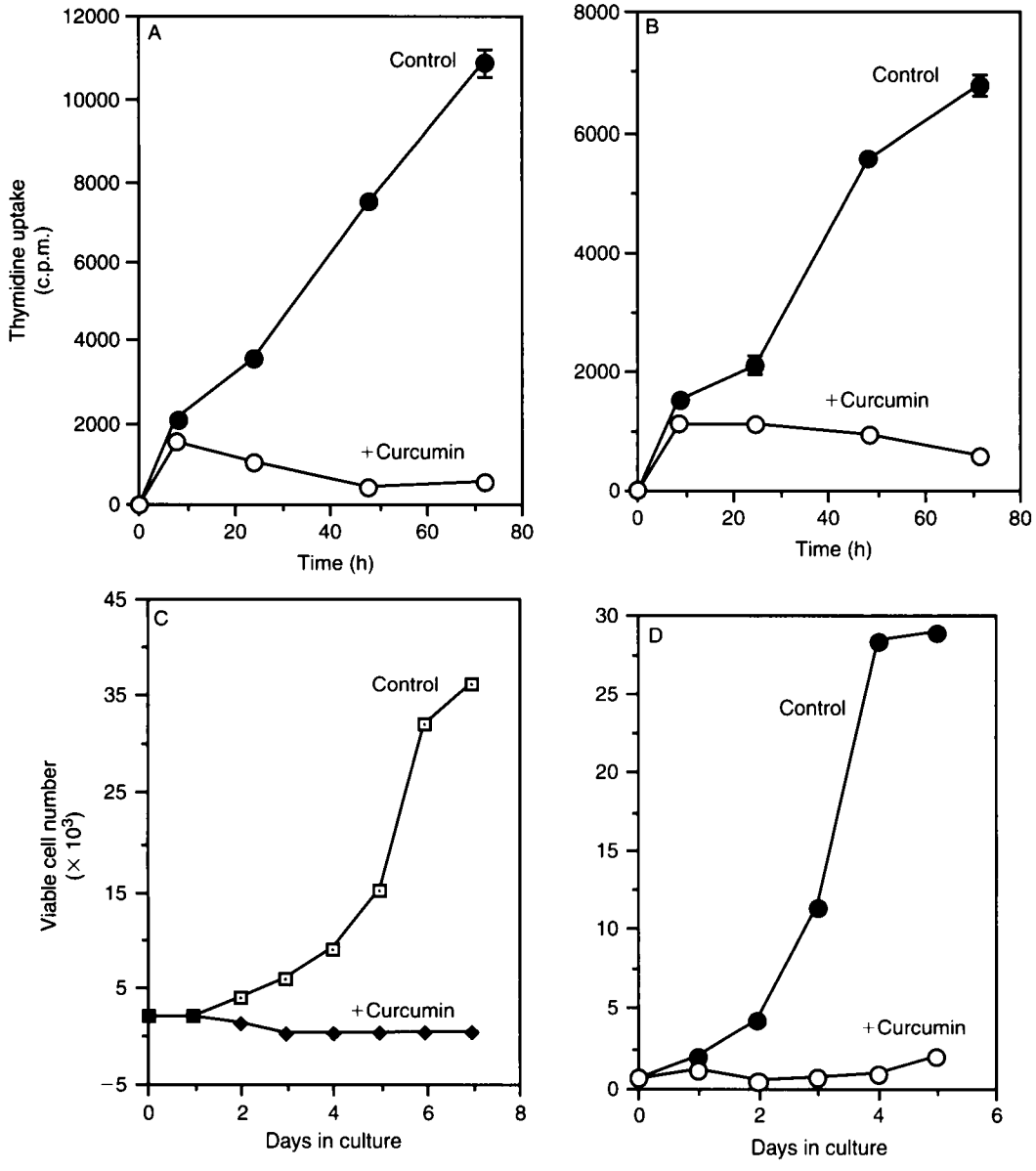

Figure 2. Time-dependent growth inhibition of human breast adenocarcinoma cells, MCF-7 (A and C), BT-20 (B) and MDA-231 (D) by curcumin. Cells (5×10^3 cells/0.2 ml/well) were plated overnight at 37°C, washed and then incubated with different concentrations of curcumin for indicated times. Viability of cells was examined either by thymidine incorporation (A and B) or by counting viable cells (C and D). For thymidine incorporation, 0.5 μCi [^{3}H]thymidine was added to the culture during the last 6 h of the incubation. The cells were washed, harvested and monitored for incorporation as described in Materials and methods. All determinations were made in triplicate. The results are expressed as percentage of the control (untreated cells).

well in 6-well plates in medium alone or medium containing increasing amounts of curcumin. The plates were incubated for 7 days to allow individual cells to form colony forming units (c.f.u.). At the end of incubation period, the number of c.f.u. were scored after staining the plates with amino black. Results of this experiment are shown in Figure 4. The majority of cells grew into c.f.u. after 7 days of culture in medium alone. However, the simultaneous presence of curcumin during the incubation period caused a dose-dependent decrease in the number of c.f.u.. The minimum effective concentration that resulted in a significant decrease in c.f.u. was 0.13 μg/ml and 1 μg/ml concentration of curcumin resulted in more than 90% inhibition in c.f.u. (Figure 4). These results suggested that the growth inhibi-

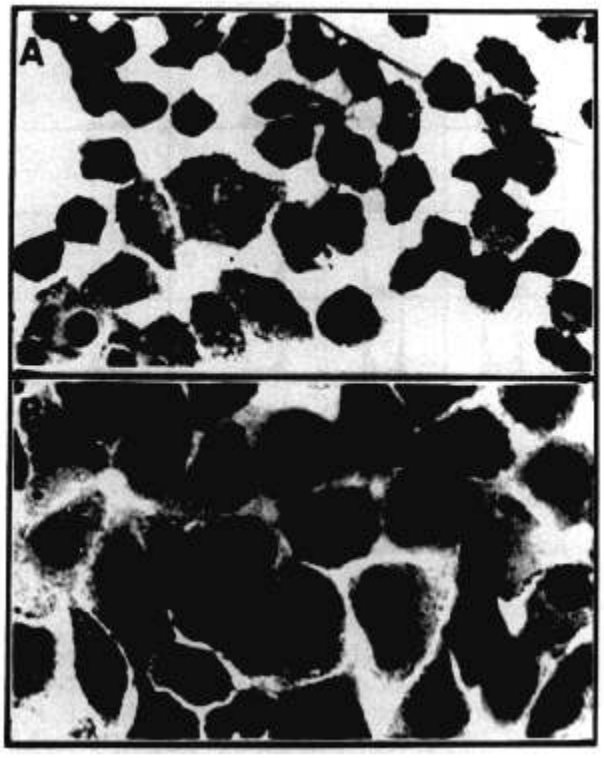

Figure 3. Effect of curcumin treatment on the morphology of MCF-7 cells. Cells cultured on glass cover slips were left untreated (A) or incubated with 2.5 μg/ml concentration of curcumin (B) for 3 days. At the end of the incubation period, the cell monolayers were washed with PBS, fixed in 100% methanol and stained with Diff-Quick stain (Dade Diagnostic, Aguada, Puerto Rico).

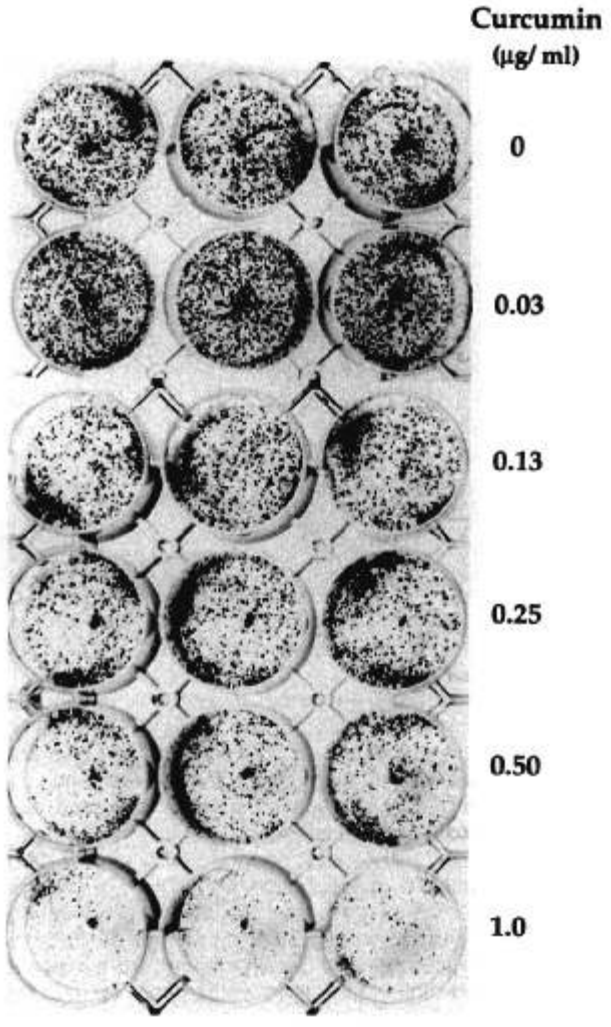

Figure 4. Dose-dependent inhibition of anchorage-dependent c.f.u.s of MCF-7 cells by curcumin. Cells were plated overnight at 37°C in 6-well plates (5×10^3/well/3 ml), washed and then incubated with different concentrations of curcumin for 72 h. At the end of incubation period, cell cultures were washed and stained with amido black.

tory effect of curcumin against breast tumor cells was cytotoxic rather than cytostatic.

Optimum antiproliferative effects require continuous presence of curcumin

To determine the minimum time required for curcumin to induce a growth inhibitory signal, we incubated MCF-7 cells with the drug for various lengths of time. At the end of each incubation period, the cells were washed and reincubated in drug-free medium for a total period of 72 h and cell growth was determined by [^{3}H]thymidine incorporation. The results shown in Figure 5 reveal that a minimum of 3 h preincubation with the drug was essential to produce a small but significant growth inhibition of MCF-7 cells. Twenty-four hour treatment with curcumin induced maximum inhibition, suggesting that the presence of curcumin for at least 24 h is needed for optimum antitumor effect against MCF-7 cells.

Curcumin blocks cell growth at the G_2/S phase of the cell cycle

We next determined if growth inhibitory effects of curcumin could be accounted for by effects on regulatory cell cycle check points. Asynchronously growing MDA-231 cells were incubated in the presence or absence of increasing concentrations of curcumin. On different days of treatment, cell fractions were analyzed for the relative DNA content by flow cytometry. The data showed that untreated cells were evenly distributed in the G_1 and S phases of the cell cycle (Figure 6A, histograms A, G, M and

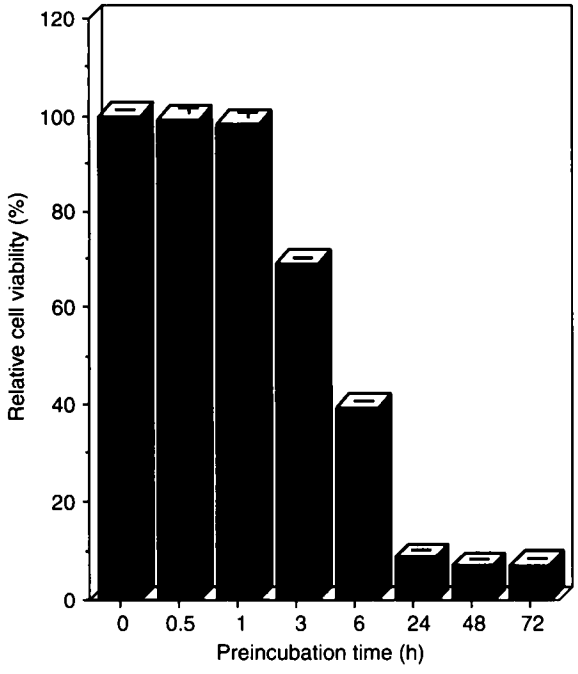

Figure 5. Continuous presence of curcumin is required to inhibit the growth of MCF-7 cells. Cells (5×10^3 cells/0.1 ml/well) were plated overnight at 37°C, washed and then incubated with curcumin (1 μg/ml) for indicated time periods. At the end of each incubation, curcumin was removed by washing, the cells resuspended in curcumin-free medium and incubation continued for a total of 72 h. During the last 6 h of incubation, 0.5 μCi [^{3}H]thymidine was added to the culture, and the cells were washed, harvested and monitored for incorporation as indicated in Materials and methods. All determinations were made in triplicate. The results are expressed as percentage of the control (untreated cells).

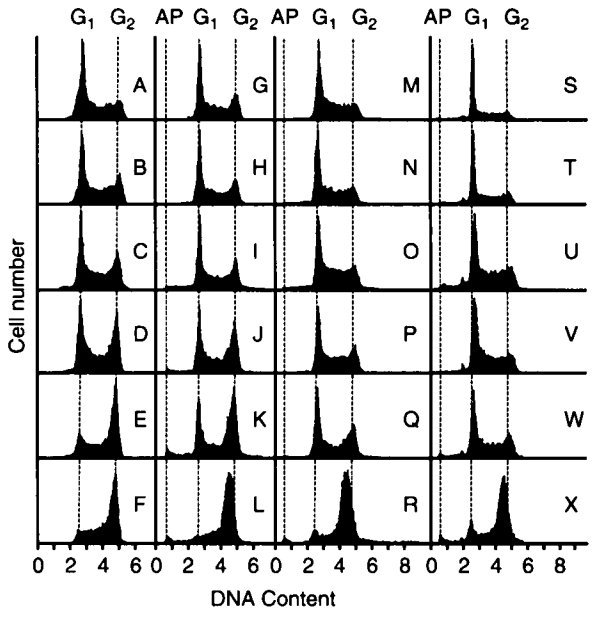

Figure 6. Growth arrest of curcumin-treated MDA-231 cells in G_2/S phase of cell cycle. Culture of MDA-231 cells were treated with curcumin at 1 (histograms B, H, N and T), 2 (histograms C, I, O and U), 3 (histograms D, J, P and V), 4 (histograms E, K, Q and W) or 5 μg/ml (histograms F, L, R and X) concentration for 1 (histograms A–F), 2 (histograms G–L), 3 (histograms M–S) or 4 days (histograms S–X). Cells from curcumin-treated or control cultures were analyzed by flow cytometry for relative DNA content, as described in Materials and Methods. $G_1 = G_0 + G_1$ cells; $G_2 = G_2 = M$ cells; AP = apoptotic cells.

S). However, treatment with curcumin caused a significant increase in G_2 checkpoint arrest (Figure 6, histograms F, L, R and X), with more than 75% cells arrested in G_2/S by 24 h after treatment (Figure 6, histograms E and F; and Table 2). The extent of G_2 checkpoint arrest was concentration dependent; at 3 μg/ml or higher concentration, the curcumin induced rapid and pronounced growth arrest in MDA-231 cells (Figure 6, vertical histograms of any day; and Table 2). Curcumin-induced cell growth arrest in G_2 was reversible with time in culture (Figure 6, horizontal histograms for any concentration). For example, the difference between curcumin-treated and untreated cells became obvious at 24 h after treatment (Figure 6, histograms A versus histograms C–E), but at later time points, cell cycle profiles of the curcumin-treated cells were virtually indistinguishable from that of the untreated asynchronously growing cells (Figure 6, histogram S versus histogram U–W), suggesting that the cells had successfully re-entered the cell cycle. At higher concentrations (5 μg/ml), curcumin appeared to cause the cells to arrest in late S phase of the cell cycle (Figure 6, histograms F, L, R and X).

Table 2. Stage-specific growth arrest of MDA-231 cells after treatment with curcumin

Curcumin (μg/ml)	Treatment time (days)	Cell cycle stage (%)[a]			
		G_1	S	G_2	Apoptosis
None	1 (A)	43	48	8	1
	4 (S)	49	38	6	7
1.0	1 (B)	34	51	14	1
	4 (T)	41	43	8	8
3.0	1 (D)	25	48	25	2
	4 (V)	33	56	3	8
5.0	1 (F)	2	45	51	2
	4 (X)	9	25	59	6

[a]Percentage of cells in various phases of cell cycle were calculated from the histograms (shown in parentheses) of Figure 5.

Curcumin-induced growth arrest is associated with ODC inhibition

Treatment of MCF-7 cells with curcumin caused a dose- and time-dependent inhibition in ODC activity (Figure 7). At a low concentration of 2.5 μg/ml, a significant decrease in ODC activity was observed 2 h after treatment, but by 24 h after treatment the cells had fully recovered from this inhibitory effect and the level of the enzyme activity was comparable to that observed in untreated cells (Figure 7A). At a higher concentration of 10 μg/ml, curcumin treatment caused a rapid and nearly complete inhibition of ODC activity that persisted even 24 h after the treatment (Figure 7B).

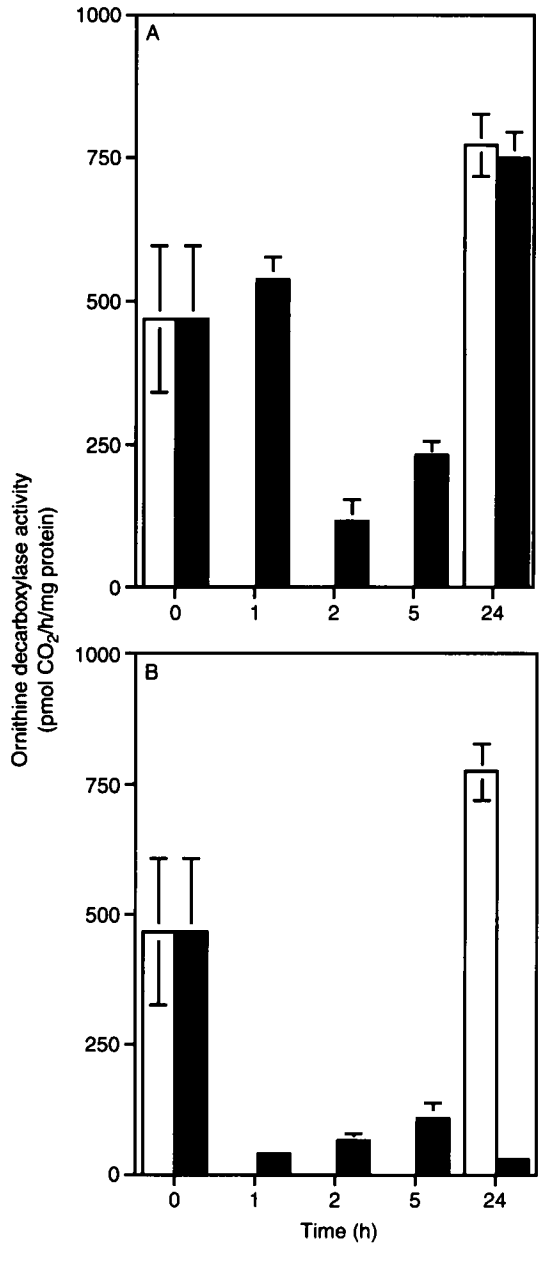

Figure 7. Effect of curcumin treatment on ODC activity in MCF-7 cells. Cells (8×10^6) were incubated in medium alone (hollow bars) or medium containing either 2.5 (A) or 10 μg/ml (B) curcumin (filled bars). At indicated time intervals, the cells were washed and processed for assaying the ODC activity as described in Materials and methods. The results shown are the averages from triplicate values ± SD from the mean.

Curcumin can overcome resistance to adriamycin in MCF-7 cells

We extended our initial observation regarding the growth inhibitory effect of curcumin to the adriamycin-resistant breast tumor cell line MCF-7_{ADR} (Table 1). This drug-resistant subclone of MCF-7 cells was selected by continuous culture in the presence of increasing concentrations of adriamycin over a period of time. The establishment and characteristics of this cell line have been described elsewhere.[24] MCF-7_{ADR} cells exhibit a 100- to 120-fold increase in resistance to adriamycin and express high levels of p-glycoprotein and TGase.[24] Unlike their differential sensitivity towards adriamycin, both the resistant and sensitive MCF-7 cells showed almost equal susceptibility to curcumin-induced growth inhibition (Figure 8).

To examine further the mechanism by which curcumin inhibits growth and to determine if drug-resistance in MCF-7 cells reflects their general resistance to apoptosis, we evaluated the levels of several proteins whose expression is modulated during apoptosis. Some striking differences in the levels of Bcl-2, p53 and TGase expression became apparent in drug-sensitive and -resistant cells (Figure 9). For instance, Bcl-2 was over produced in sensitive cells as compared to resistant cells, whereas p53, and TGase were produced only in the adriamycin-resistant cells. Treatment with curcumin of either adriamycin-sensitive or -resistant cells failed to alter the production of any of these proteins, suggesting that curcumin-mediated growth inhibition of resistant cells may operate by a mechanism other than apoptosis. Consistent with these results are those obtained by flow cytometry data (Table 2 and Figure 6), morphological alterations (Figure 3) and DNA laddering data (not shown) which showed no significant increase in the proportion of cells undergoing apoptosis even after prolonged treatment with curcumin.

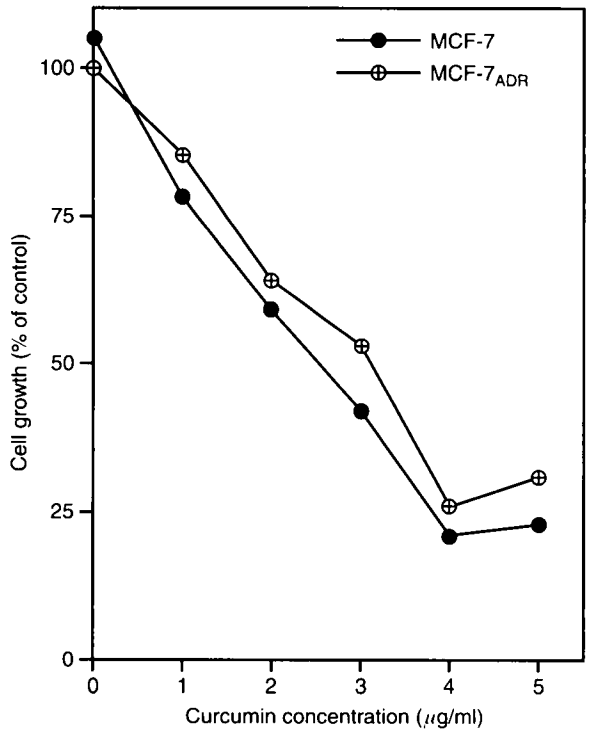

Figure 8. Effect of curcumin treatment on cell growth of drug-sensitive and adriamycin-resistant MCF-7 cells. Cells (5×10^3/well/0.2 ml) were grown overnight in 96-well plates and then treated with increasing concentrations of curcumin for 3 days. The number of surviving cells at the end of incubation was determined by MTT assay as described in Materials and methods.

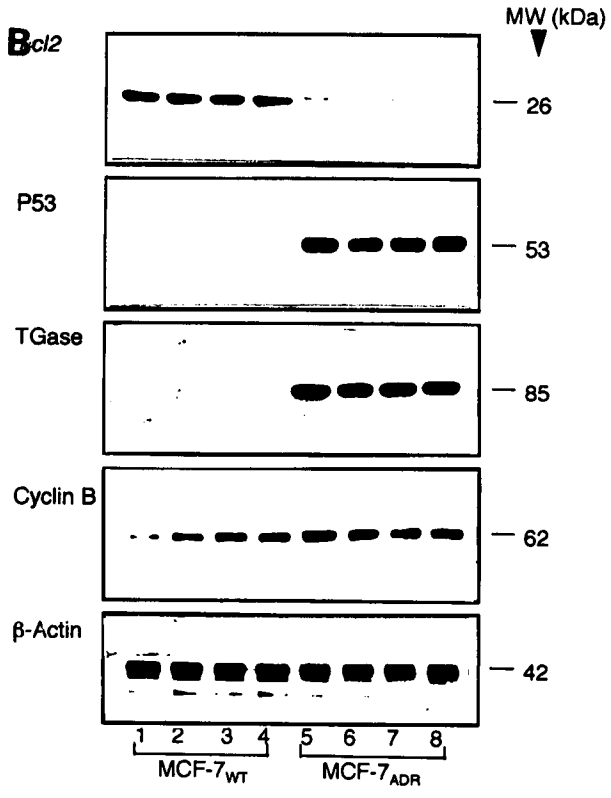

Figure 9. Effect of curcumin treatment on apoptosis-related proteins in MCF-cells. Drug-sensitive (lanes 1–4) and adriamycin-resistant (lanes 5–8) MCF-7 cells were incubated in medium alone (lanes 1 and 5) or medium containing 2.5 μg/ml curcumin (lanes 2, 3, 4, 6, 7 and 8) for 24 (lanes 2 and 6), 48 (lanes 3 and 7) and 72 h (lanes 4 and 8). At the end of incubation period, cells were washed, lysed and subjected (50 μg protein) to Western blotting by using specific antibodies as primary antibody and horseradish peroxidase-conjugated anti-mouse IgG as secondary antibody. The antigen–antibody reaction was detected by ECL as described in Materials and methods.

Discussion

The search for new chemopreventive and antitumor agents that are more effective and less toxic has kindled great interest in phytochemicals. Curcumin which is derived from the root of a plant, *C. longa*, is one such compound. It has been used as a dietary factor and as a herbal medicine for centuries in several Asian countries. In the present report, we describe the potent antiproliferative effects of curcumin against a wide variety of breast tumor cell lines. The antiproliferative effects were observed against hormone-independent and -dependent and adriamycin-sensitive and -resistant breast tumor cells. The antiproliferative effects of curcumin were observed at concentrations of 2–15 μM which have been shown to inhibit PKC activity *in vitro*, EBV-DR promoter, SV 40 promoter enhancer, AP-1 activation, NF-κB activation and HIV-LTR-mediated transcription.[16,17,28,29] Normal cells were relatively resistant to the toxic effects of curcumin (data not shown), suggesting that the growth inhibitory effects of this drug against tumor cell lines were specific.

How different antiproliferative agents suppress cellular growth is not well understood. Certain cytokines such as interferon-α, interleukin-6 and transforming growth factor-β inhibit cellular proliferation by inducing tumor suppressor genes such as retinoblastoma (Rb) or by suppressing the expression of genes involved in cellular proliferation or by modulating the phosphorylation of the gene product.[30,31] Since phorbol ester-mediated tumor promotion is inhibited by curcumin and this was shown to be due to the inhibitory effects of curcumin on protein kinase C,[18] it is possible that the growth-inhibitory effects of curcumin are also

mediated through its ability to inhibit specific protein kinases. Our studies clearly suggest that curcumin treatment results in growth arrest of breast tumor cells in the G_2 phase of the cell cycle (Figure 6 and Table 2). In higher eukaryotes, several cyclin-dependent kinases (Cdks) play a crucial role during cell proliferation. For example, at G_2/M phase transition, mitosis is initiated by a Cdk–cyclin complex that is composed of a Cdc2 protein kinase and a B-type cyclin. Cdc2 kinase phosphorylates a variety of cellular proteins, including the tumor suppressor gene products Rb and p53 that regulate critical events during cell growth.[32,33] It is likely that curcumin affects the growth of tumor cells by inhibiting the activity of Cdc2 or some other kinases that have a role in initiating mitosis. We have previously reported that curcumin can inhibit both protein tyrosine kinases and serine/threonine protein kinases *in vitro*.[21] Other investigators demonstrated that curcumin could also inhibit *in vivo* the intrinsic kinase activity of the epidermal growth factor (EGF) receptor,[22] which may result in anti-growth action of curcumin. In addition, curcumin was also shown to inhibit EGF-induced activation of EGF receptor phosphorylation.[23]

In general, G_2 arrest of proliferating cells is caused by DNA-damaging agents and represents a mechanism of negative feedback control for the induction of gene products that facilitate the repair of DNA lesions. Therefore, G_2/M phase arrest is thought to ensure that DNA replication will proceed with fidelity and to avoid segregation of defective chromosomes.[34] If DNA repair is successful during G_2 phase, the cells re-enter the cell cycle; otherwise, they are eliminated via apoptosis. Our studies suggested that curcumin treatment causes G_2/M arrest of the human breast tumor cells without any evidence of apoptosis (Table 2). Indeed, the tumor cells rescued the growth inhibitory effect with time in culture (Figure 6). The recovery from growth arrest depended on the concentration of curcumin used. Moreover, curcumin treatment did not alter the expression of the apoptosis-related proteins Bcl-2, p53 and TGase (Figure 9).

ODC, a key regulatory enzyme in polyamine biosynthesis, was strongly inhibited by curcumin (Figure 7). At lower concentrations, curcumin transiently inhibited ODC activity in MCF-7 cells. However, at higher concentrations the inhibition was acute and persisted for at least 24 h after treatment. The ability of curcumin to inhibit ODC activity correlated well with its growth inhibitory activity. Cells treated at lower concentrations of curcumin were able to escape more rapidly from G_2 arrest than those treated at higher concentrations (Figure 6, horizontal groups of histograms). ODC plays a pivotal role in cell growth and proliferation since disruption of its functions by agents such as DFMO results in growth arrest.[35,36] Overexpression of ODC has also been linked to cell transformation and carcinogenesis. Most transformed cell lines and tumors exhibit high levels of basal ODC expression, and its induction has been suggested to be a critical event for tumor promotion in a variety of experimental models including skin, breast and colon carcinogenesis.[37] From these studies, it is tempting to speculate that anticarcinogenic properties of curcumin may be attributed to its ability to inhibit ODC activity.

Another interesting feature of this study was the observation that curcumin could circumvent the drug-resistant phenotype in breast tumor cells. Curcumin was as effective in arresting the growth of multidrug-resistant and TNF-resistant cells as it was wild-type MCF-7 cells (Table 1 and Figure 8). These studies suggested that curcumin does not serve as a substrate for P-glycoprotein and that it uses unique targets in resistant cells. Previous studies have demonstrated elevated levels of PKC activity in multi-drug resistant MCF-7 cells.[38] The agents that alter PKC activity have been shown to modulate the drug-resistant phenotype.[39] It is likely that curcumin mediates its effects against drug-resistant cells by virtue of its ability to inhibit PKC. Furthermore, the growth inhibitory effects of certain cytokines and chemotherapeutic agents correlate with their ability to modulate intracellular glutathione levels.[40,41] The inhibitory effect of curcumin against PKC activity is suppressed by thiol compounds such as mercaptoethanol, cysteine and dithiothreitol.[18] Whether the sensitivity of tumor cells and resistance of normal cells to curcumin is dependent on cellular glutathione levels is not clear.

Previously we reported that curcumin is a potent inhibitor of NF-κB activation induced by a wide variety of agents including TNF, phorbol ester and hydrogen peroxide.[17] Therefore, it is equally possible that the target for curcumin action in transformed cells could lie upstream in the signal transduction pathway involving NF-κB, which serves as a central coordinating regulator in signal-responsive transduction of several genes, including c-*myc* and p53, which in turn play important roles in cell growth, differentiation, activation and chemotaxis.[42] The absence or inhibition of NF-κB in normal or transformed cells has recently been shown to render them more sensitive to chemotherapy, radiation and TNF-induced apoptosis.[43–47] Thus, it is conceivable

that the anti-proliferative effect of this compound may be linked to this pathway. In any case, the low toxicity at pharmacological doses and potent anti-proliferative activity against several breast tumor cell lines exhibiting various phenotypes lead us to conclude that the therapeutic potential of curcumin in breast cancer warrants further investigation.

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