

Original Article

The cellular uptake and cytotoxic effect of curcuminoids on breast cancer cells

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Abstract

Objective: Curcuminoids (including curcumin) are natural antioxidants demonstrating potent chemopreventive properties against several forms of cancer. This study investigated the antiproliferative and induced apoptotic effects of curcuminoids on three cell lines isolated from human breast adenocarcinoma and ductal carcinoma (MDA-MB-231, MDA-MB-435S, and MCF-7).

Materials and Methods: This study developed a highly sensitive, reproducible assay method using high-pressure liquid chromatography to quantify the cellular uptake of curcuminoids by breast cancer cells and quantitate its effect on inhibition of proliferation and activation of apoptosis in breast cancer cells.

Results: Results indicate that curcuminoids inhibited cell proliferation and activation of apoptosis in the cell lines in this study. Both effects were observed to increase in proportion to the cellular uptake of curcuminoids; cellular uptake increased following an increase in the dosage of curcuminoids.

Conclusion: The inhibition of proliferation and increased apoptosis of breast cancer cells appears to be associated with the uptake of curcuminoids by cancer cells.

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Keywords: apoptosis; breast cancer; cellular uptake; curcuminoids

Introduction

Turmeric (a curcuminoid) is a herb isolated from the rhizome of *Curcuma longa*. It is a popular spice in oriental dishes such as curry and a drug found in traditional medicines throughout Asia [1–3]. Turmeric has attracted considerable interest in recent years due to its putative health benefits as an antioxidant [4] and anti-inflammatory [5] agent, as well as for its treatment potential in the prevention of cancer [6,7].

A variety of mechanisms have been proposed to explain the anticarcinogenic activity of curcuminoids: antioxidantizing and anti-inflammatory properties, induction of phase II detoxification enzymes, inhibition of cyclooxygenase 2, an influence on the transcription factors activator protein 1 (AP-1) and nuclear factor kappa B, inhibition of matrix metalloproteinase, and effects on protein kinases as well as other effects [8,9]. This study investigates the inhibition of cell proliferation and the induction of apoptosis by curcumin to gain insight into the underlying mechanisms associated with the chemopreventive effects of curcuminoids.

Most of the various methods used for the detection and analysis of curcuminoids involve the use of spectrophotometry to characterize the yellow color in the samples [10–12]. Commercially available products are generally a mixture of

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the primary ingredient, curcumin, with demethoxycurcumin (DMC) and bisdemethoxycurcumin (bDMC). Researchers have been unable to quantify the individual components of curcuminoids using spectrophotometric methods; however, liquid chromatography–mass spectrophotometry and radio-labeling have recently been successfully applied to the detection of curcumin [13–15].

This paper reports an investigation into whether curcuminoids contribute to antiproliferation and apoptosis in human breast cancer cell lines: two of breast adenocarcinoma and one of ductal carcinoma. We also sought to determine whether these effects could be explained qualitatively and quantitatively, according to the bioavailability of the curcuminoids. To achieve this, we developed a highly sensitive, reproducible assay method using high-pressure liquid chromatography (HPLC) to quantify the cellular uptake of curcuminoids by breast cancer cells.

Materials and methods

Cell lines and culture conditions

The cell lines used in this series of investigations – MDA-MB-435S (a metastatic human ductal carcinoma with no tumorigenic in nude mice; ATCC HTB-129), MDA-MB-231 (a poorly differentiated human breast adenocarcinoma grade III, with extracellular growth factor receptors, transforming growth factor alpha receptors, and *WNT7B* oncogene expression, with tumorigenic in nude mice; ATCC HTB-26) and MCF-7 (a human breast adenocarcinoma, with estrogen receptors and *WNT7B* oncogene expression; ATCC HTB-22) – were obtained from ATCC (Manassa, VA, USA) and maintained in culture medium (GibcoBRL, USA) based on the specifications provided by ATCC. The medium was supplemented with 10–15% (v/v) fetal bovine serum (HyClone, Logan, UT, USA) and a combination of antibiotics (HyClone) with or without an atmosphere of carbon dioxide/air (5%).

Assay of cell proliferation

The proliferation of the breast cancer cell lines after exposure to curcuminoids was measured by the CellTiter96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA). Briefly, the cells were first incubated with curcuminoids for 24 hours and then treated with MTS [3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium] for 4 hours. Absorbance was determined using a PowerWave XS Reader (Bio-tek, VT, USA) at 490 nm. Each assay was carried out in triplicate and the results were expressed as the mean \pm standard error of the mean (SEM). Cell proliferation was expressed as the percentage of the assay data determined for the control group.

Assay of cell apoptosis

The apoptosis was assessed by the APOPercentage Apoptosis Assay kit (Biocolor Ltd., Carrickfergus, Antrim,

UK) and Annexin V-FITC Apoptosis Detection Kit (Strong Biotech, Taipei, Taiwan). Briefly, the breast cancer cells (5×10^4) were seeded in the culture medium, which contained 100 μ L of serum, and dispensed into 96-well microplates. After the culture medium had been treated with curcuminoids for 24 hours, it was replaced with a fresh medium plus the APOPercentage Dye Label. The APO% Dye Release Reagent was then added to each well to aid cell lysis and the release of bound dye from the apoptotic cells. The amount of the cell-bound dye recovered in the solution was measured using the PowerWave XS Reader. A reference wavelength of 655 nm was used to estimate the apoptotic index at the wavelength of 550 nm. Each assay was carried out in triplicate, and the results were expressed as the mean \pm SEM of the absorption at 550 nm.

The apoptotic cells were also detected using an Annexin V-FITC Apoptosis Detection Kit and inspected by a Fluorescence Microscopy CKX41 and U-RFLT 50 (Olympus, Australia). The filter for FITC was BA520 IF (BP460-490C) and propidium iodide was BA590 (BP480-550C).

Evaluation of mitochondrial membrane potential

The breast cancer cells were first seeded in 24-well plates (Orange Scientific, CA, USA) for 16–24 hours. Following treatment with curcuminoids, rhodamine 123 (10 μ g/mL; Sigma, CA, USA) was added to the culture medium, 50 μ L per well, and then incubated (at 37 °C for 20 minutes) for mitochondrial staining. After washing twice with warm phosphate buffered saline, the cells were fixed with 2% paraformaldehyde and inspected using fluorescence microscopy (Olympus CKX41 and U-RFLT 50).

Recovery of curcuminoids from breast cancer cells for assay

The cell pellets were each suspended in the RIPA buffer [20 mM Tris/HCl (at pH 8.0), 5 mM EDTA, 1 mM phenylmethyl-sulfonyl fluoride, 1.5 mg leupeptin, 137 mM NaCl, 10% (v/v) Glycerol, and protease inhibitor cocktail], and cell–liquid extraction was then carried out immediately. The cell extracts were centrifuged at 14,000 g for 15 minutes, and the amounts of total cellular protein in the extracts were determined using the Bradford method (Bio-Rad, CA, USA). The cell extract obtained was acidified, 100 μ L each, using 6 N HCl (1:1 w/v) and vortexed for 30 seconds. A further volume of extracting solution (500 μ L) was then added to each of the acidified cell extracts. Samples were again centrifuged and then shaken in the Orbited shaker (Heng-Chen, Kaohsiung, Taiwan) (at 100 rpm) for 15 minutes. After centrifugation at 18,000 rpm for 20 minutes, the upper organic layer was filtered using a membrane filter (0.22 μ m) and transferred to a clean injection sample vial (approximately 100 μ L) for a quantitative analysis using the Recovery curcuminoids and high performance liquid chromatography (HPLC) methods previously described [7].

A Hitachi L-2000 series HPLC system (Hitachi, Osaka, Japan) was used to carry out the HPLC analysis of

curcuminoids; this consisted of a solvent delivery system (L-2130 model) equipped with a fluorescence detector (L-2480 model), an autosampler (L-2130 model), and a reversed-phase column (Purospher STAR RP-18e, 5mM; Merck, NJ, USA) maintained at 37 °C by a column oven (L-2350 model). A mobile phase, which was composed of acetic acid (2%) pH 2.5 and acetonitrile (in a ratio of 47:53) and delivered at a flow rate of 0.8 mL/min, was used, while the samples were each injected with an injection volume of 20 μ L. With the use of an excitation wavelength of 420 nm and an emission wavelength of 540 nm, curcumin, DMC, and bDMC were detected [7].

Statistical analysis

All data are reported as the mean \pm SEM of at least three separate experiments. Statistical analysis was carried out using a *t* test, with significance set at $p < 0.05$.

Results

This investigation was based on the hypothesis that curcuminoids could affect the survival of breast cancer cells, inhibiting their proliferation. To evaluate our hypothesis and explore the antitumor effects of curcuminoids on breast cancer cells, we performed an *in vitro* study in which three breast cancer cell lines were treated with increasing dosages of curcuminoids (0, 12.5, 25, and 50 μ M) over a period of 24 hours. The proliferation of cancer cells treated with curcuminoids was then determined using MTS. The results summarized in Fig. 1A indicate a decrease in the survival and proliferation of breast cancer cells following an increase in dosage of curcuminoids. Moreover, the inhibition of proliferation was found to vary according to the type of breast cancer: breast cancer cells from cell lines MDA-MB-231 and MDA-MB-435S were more sensitive to the antiproliferative action of curcuminoids than those from the MCF-7 cell line following exposure to 25–50 μ M curcuminoids for 24 hours.

To explore whether the anti-proliferative action of curcuminoids was the result of apoptosis or necrosis of the breast cancer cells, we used the APOPercentage Apoptosis Assay kit to identify the formation of apoptotic cells in the three breast cancer cell lines following exposure to curcuminoids for 24 hours. The results of APOPercentage Apoptosis assay are shown in Fig. 1B, in which the pink-colored deposits are a positive indication of the existence of apoptotic cells. Measuring the absorbance at 550 nm enabled quantification of the apoptotic cells in each of the breast cancer cell lines. The results summarized in Fig. 1C suggest that the increase in apoptosis of the breast cancer cells was dose-dependent, such that the higher the dose of curcuminoids (0, 12.5, 25, or 50 μ M), the greater the extent of apoptosis (Fig. 1C). The quantitative increase in induced apoptosis was observed in all three cell lines. The data in Fig. 1C also suggest that, following a fourfold increase in the dose of curcuminoids (from 12.5 μ M to 50 μ M), induced apoptosis increased from a twofold to a fourfold enhancement of activation over the control (all data statistically significant at $p < 0.05$).

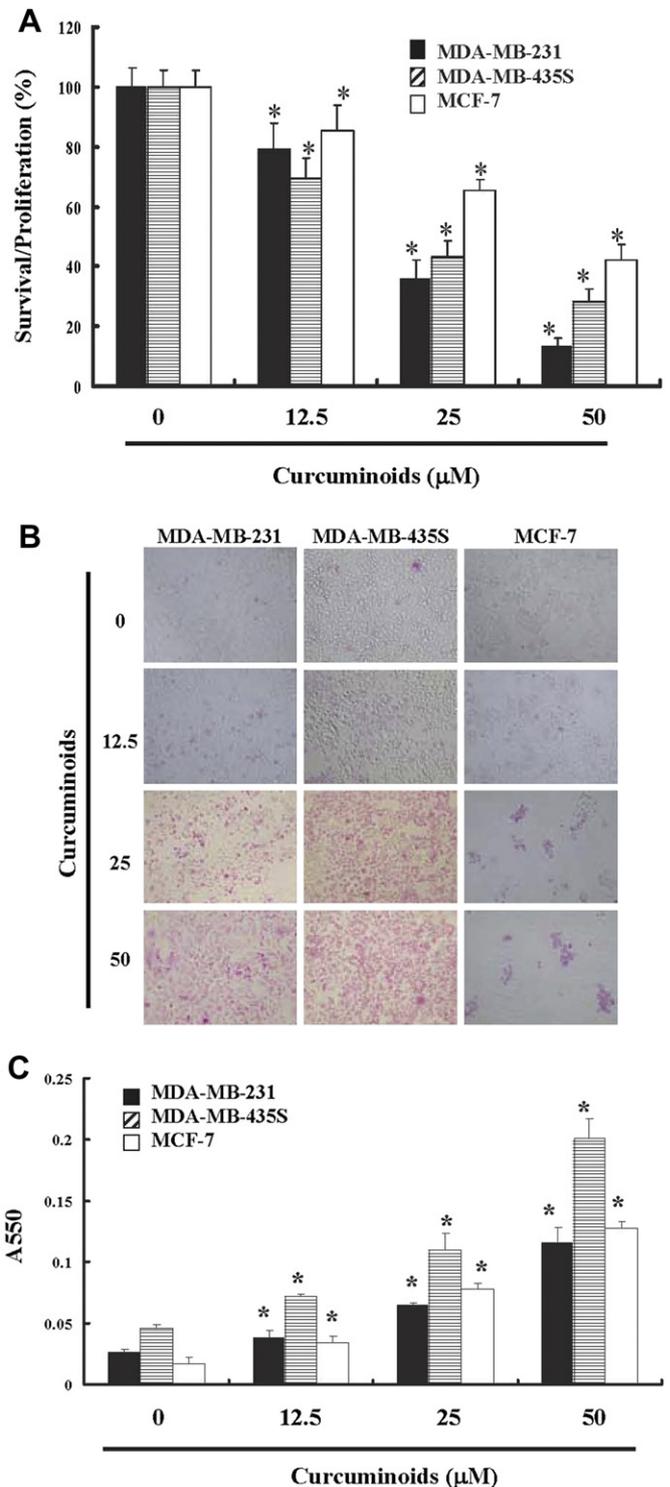


Fig. 1. Inhibitory effect of curcuminoids on the survival/proliferation of human breast cancer cells. (A) Three lines of human breast cancer cells (MDA-MB-231, MDA-MB-435S, and MCF-7) were used in the study. The cell lines were each treated with an increasing dose (0, 12.5, 25, and 50 μ M) of curcuminoids ($n = 6$ for each study group), and the levels of survival and proliferation were assessed by MTS assay. The solid bars (MDA-MB-231 cells), hatched bars (MDA-MB-435S cells), and open bars (MCF-7 cells) represent the levels of cell survival and proliferation, as a percentage of the control values (controls being treated with 0 μ M curcuminoids). (B, C) Apoptosis of breast cancer cells in the three cell lines (MDA-MB-231, MDA-MB-435S, and MCF-7) induced by curcuminoid treatment was determined using the APO Percentage

Analysis of the assay data in Fig. 1A and C appears to suggest that the dose-dependent inhibition of proliferation in the breast cancer cells could be attributed to apoptosis induced by treatment with curcuminoids.

We used rhodamine to investigate whether the mitochondrial membrane potential (MMP) in the breast cancer cells was affected by treatment with curcuminoids. The results in Fig. 2A indicate that the MMP of breast cancer cells was reduced following treatment with curcuminoids (up to 50 μM), which demonstrates that the degree to which MMP was reduced depends on the dosage of curcuminoids to which the breast cancer cells were exposed.

Curcuminoid-induced apoptosis was further evaluated by staining the breast cancer cells with Annexin V-FITC and propidium iodide following treatment with 25 μM curcuminoids. The apoptotic cells were then examined under a fluorescence microscope, the results indicating that treatment with curcuminoids induced apoptosis in breast cancer cells, as shown in Fig. 2B.

The results shown in Figs. 1 and 2 led us to the conclusion that the inhibition of proliferation in all three breast cancer cell lines was the result of apoptosis induced by curcuminoids.

To characterize the kinetics involved in the uptake of curcuminoids by the breast cancer cells, we developed a reproducible HPLC method with a high degree of sensitivity to quantify the level of curcuminoids taken up by the cancer cells following treatment. Using the HPLC system under the conditions outlined in the Methods section, the major components of curcuminoids, curcumin (the principal constituent), and DMC and bDMC (its demethoxylated derivatives), yielded three well-defined chromatographic peaks (Fig. 3A). Further analysis indicated that the area under the peaks associated with curcumin yielded perfect linearity with the concentrations of curcuminoids used in the treatment (with a correlation coefficient of 0.96). These results were produced from a series of six standard solutions containing curcuminoids in the concentration range 0–80 μM . Similar linearity was also observed in the peak areas associated with DMC and bDMC (with correlation coefficients of 0.9825 and 0.9608, respectively; data not shown). These results indicate the applicability of the HPLC method developed in this study for quantitatively assaying curcumin and its demethoxylated derivatives (DMC and bDMC).

This study also evaluated the efficiency and precision of the extraction method developed for the recovery of curcuminoids. We analyzed the results of recovery obtained through repeating the extraction of curcuminoids at three concentration levels: high (50 μM), medium (25 μM), and low (12.5 μM).

Apoptosis Assay kit. The kit identifies the apoptotic cells as the pink-colored deposits in the cultures of human breast cancer cells (B) after exposure to an increasing dose (0, 12.5, 25, and 50 μM) of curcuminoids for 24 hours. (C) Bars represent the absorbance at a wavelength of 550 nm, which is a measurement of the apoptotic cells in each of the breast cancer cell lines (solid bars, MDA-MB-231 cells; hatched bars, MDA-MB-435S cells; open bars, MCF-7 cells). Data are shown as the mean \pm SEM of at least three independent experiments. * Difference from controls (treated with 0 μM curcuminoids) is statistically significant at $p < 0.05$.

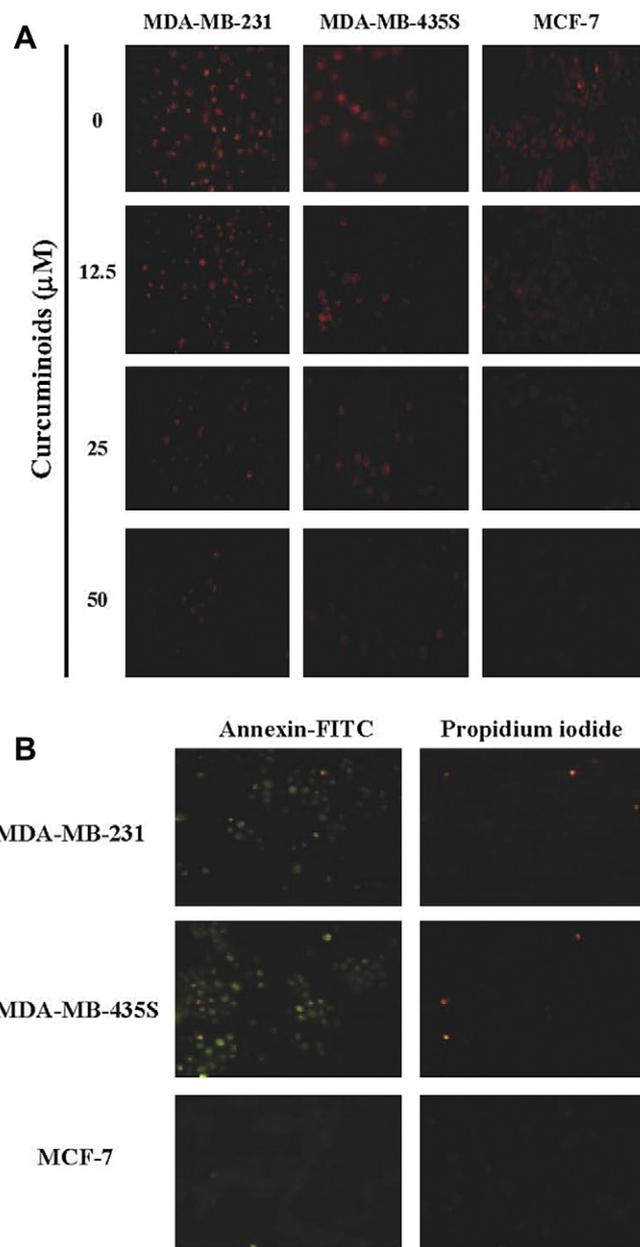


Fig. 2. Apoptosis assayed by measuring the reduction of mitochondrial membrane potential (MMP) in human breast cancer cells produced by curcuminoid treatment. (A) After rhodamine staining, MMP in the breast cancer cells was determined by measuring the intensity of red fluorescence on fluorescence microscopy. The results indicated that MMP was significantly reduced after treatment with 25–50 μM curcuminoids compared to controls (which were treated with 0 μM curcuminoids). (B) Apoptosis assayed by staining with Annexin V-FITC and propidium iodide and examination under a fluorescence microscopy after the breast cancer cell lines had been treated with 25 μM curcuminoids.

We obtained samples from the culture medium as well as from the pellets of breast cancer cells. The results indicate that the proposed extraction method achieved recovery efficiency of $72.4 \pm 5.2\%$, $67.2 \pm 5.3\%$, and $63.9 \pm 4.1\%$, respectively, for curcumin, DMC, and bDMC in the samples prepared from culture medium, as well as $67.9 \pm 5.3\%$, $65.6 \pm 4.9\%$, and $62.5 \pm 4.3\%$, respectively, for curcumin, DMC, and bDMC in the samples prepared from the cell pellets. These values of

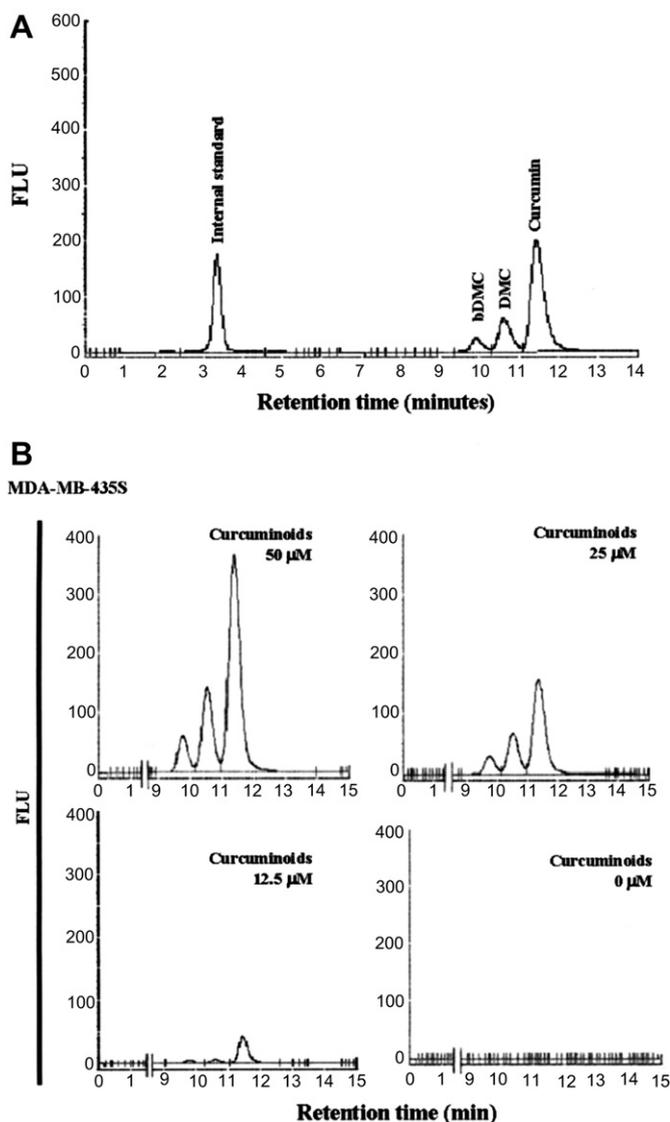


Fig. 3. HPLC chromatogram of curcuminoids taken up by human breast cancer cells and remaining in the culture medium used for cultivation of the breast cancer cells. (A) Using the HPLC conditions and extraction method developed, the characteristic peaks for the major components of the curcuminoids curcumin, demethoxycurcumin (DMC), and bisdemethoxycurcumin (bDMC) could be well differentiated and quantitated. (B) A typical set of HPLC chromatograms to illustrate the variation of the HPLC peaks for curcumin and its demethoxylated derivatives (DMC and bDMC), recovered from the breast cancer cells (of the MDA-MB-435S cell line) by the extraction method developed, in proportion to the dose of curcuminoids added into the culture medium for the 24-hour treatment of cancer cells.

recovery efficiency were then used to determine the quantity of curcumin, DMC, and bDMC recovered from the cancer cells and culture medium in the cellular uptake studies reported below.

To study the cellular uptake kinetics of curcuminoids by human breast cancer cells, we determined the levels of curcuminoids in the cancer cells (following treatment with curcuminoids for 24 hours) using the cell–liquid extraction method outlined earlier. The curcuminoid remaining in the culture medium was also extracted, using the liquid–liquid extraction method, also described earlier. Fig. 3B presents

a typical set of HPLC chromatograms for the curcuminoids recovered from breast cancer cells. The results illustrate that the HPLC analytical method effectively characterized the specific peaks for curcumin and its demethoxylated derivatives (DMC and bDMC), as obtained from samples prepared with culture medium (Fig. 3A). Furthermore, these HPLC peaks increased in height and area subsequent to an increase in the dosage of curcuminoids to which the breast cancer cells were exposed.

We calculated the recovery of curcumin, DMC, and bDMC from the breast cancer cell lines from the peak areas in Fig. 3B. The results summarized in Table 1 indicate that the amounts of curcumin, DMC, and bDMC taken up by the breast cancer cells in all three cell lines increased proportionally with the dose of curcuminoids used in the treatment. In other words, the higher the dose used, the greater the amount of curcumin, DMC, and bDMC recovered from the cancer cell pellets.

The data related to cellular uptake outlined in Table 1 also suggest that the quantities of curcumin and its demethoxylated derivatives taken up by the breast cancer cells varied according to the cell line. This implies that the total amount of curcuminoids taken up by the breast cancer cells showed a cell line-dependent trend: MDA-MB-435S > MDA-MB-231 > MCF-7.

To explore the chemoprotective potential of curcuminoids, we investigated the relationship between the inhibition of proliferation (Fig. 1A) [as well as the induction of apoptosis (Fig. 1C)] and the cellular uptake profiles of curcumin and its demethoxylated derivatives (Table 2). The results for the inhibition of proliferation are shown in Fig. 4A and those for the induction of apoptosis in Fig. 4B. According to the profiles in Fig. 4A, a linear correlation exists between the inhibition of survival/proliferation and the logarithm of the curcuminoids recovered from the cancer cells. The same correlation was

Table 1

Cellular uptake of curcuminoids by human breast cancer cells and the recovery of curcumin and its demethoxylated derivatives (DMC and bDMC) from cancer cells.

Dose (μM)	MDA-MB-231 Cells ($\mu\text{g}/\text{mg}$ total protein)		
	Curcumin	DMC	bDMC
0	ND	ND	ND
12.5	3.39 ± 0.06	ND	ND
25	6.46 ± 0.03	3.14 ± 0.08	2.91 ± 0.05
50	12.01 ± 0.10	6.78 ± 0.14	5.20 ± 0.08
Dose (μM)	MDA-MB-435S Cells ($\mu\text{g}/\text{mg}$ total protein)		
	Curcumin	DMC	bDMC
0	ND	ND	ND
12.5	4.72 ± 0.01	2.85 ± 0.02	ND
25	7.60 ± 0.04	4.38 ± 0.06	2.75 ± 0.03
50	14.68 ± 0.19	7.40 ± 0.13	4.52 ± 0.10
Dose (μM)	MCF-7 Cells ($\mu\text{g}/\text{mg}$ total protein)		
	Curcumin	DMC	bDMC
0	ND	ND	ND
12.5	2.30 ± 0.01	ND	ND
25	6.16 ± 0.02	2.24 ± 0.03	ND
50	10.87 ± 0.05	4.56 ± 0.01	2.22 ± 0.01

Data are the mean \pm SEM of at least three independent experiments. bDMC = bisdemethoxycurcumin; DMC = demethoxycurcumin; ND = non-detectable.

Table 2
Relationship between antiproliferative the concentration of 50% inhibition (IC₅₀) and cellular level of curcuminoids in human breast cancer cells.

Human Breast Cancer Cell Lines	MDA-MB-231	MDA-MB-435S	MCF-7
Anti-proliferation IC ₅₀ (μM)	17.31 ± 1.12	25.13 ± 0.87	45.47 ± 1.61
Cellular curcuminoids level* (μg/mg total protein)	4.30 ± 0.46	7.65 ± 0.89	9.96 ± 0.97

Data are the mean ± SEM of at least three independent experiments. bDMC = bisdemethoxycurcumin; DMC = demethoxycurcumin; ND = non-detectable.

* Cellular level at IC₅₀ value, which is calculated from the cellular levels (in Table 1) for each of the cell lines.

observed for each of the cell lines. These observations imply that the proliferation of breast cancer cells depends exponentially on the amount of curcuminoids taken up by the cancer cells. Further analysis indicates that the concentration of 50% inhibition (IC₅₀) values for the inhibition of proliferation are related to the cellular level of curcuminoids recovered from the cells of all three breast cancer cell lines.

By contrast, an increase in the induction of apoptosis in breast cancer cells was observed following an increase in the cellular level of curcuminoids (Fig. 4B). This observation suggests that the induction of apoptosis is linearly dependent upon the quantity of curcuminoids taken up by the cancer

cells. The same linearity was observed in all three cell lines studied.

Collectively, the data illustrated in Fig. 4A and B and Table 1 imply that the increase in the cellular uptake of curcuminoids and their accumulation in the breast cancer cells could play an important role in arrested growth and cell death.

Inhibition of cell proliferation and induction of apoptosis are regarded as key strategies in the prevention of cancer [7]. The development of rapid, reliable, sensitive HPLC assay methods to measure the cellular uptake of curcuminoids and analyze the quantitative relationship with the proliferation and apoptosis of cancer cells could be highly beneficial to researchers in breast cancer therapy.

Discussion

This review presents an overview of the current *in vitro* and *in vivo* data supporting the therapeutic benefits of curcuminoids in cancer treatment as well as the challenges concerning their development as adjuvant chemotherapeutic agents [19]. The results collected in this series of studies using the cell lines of breast adenocarcinoma and breast ductal carcinoma (isolated from breast cancer patients) provide experimental evidence that curcuminoids irreversibly induce the apoptosis of breast cancer cells.

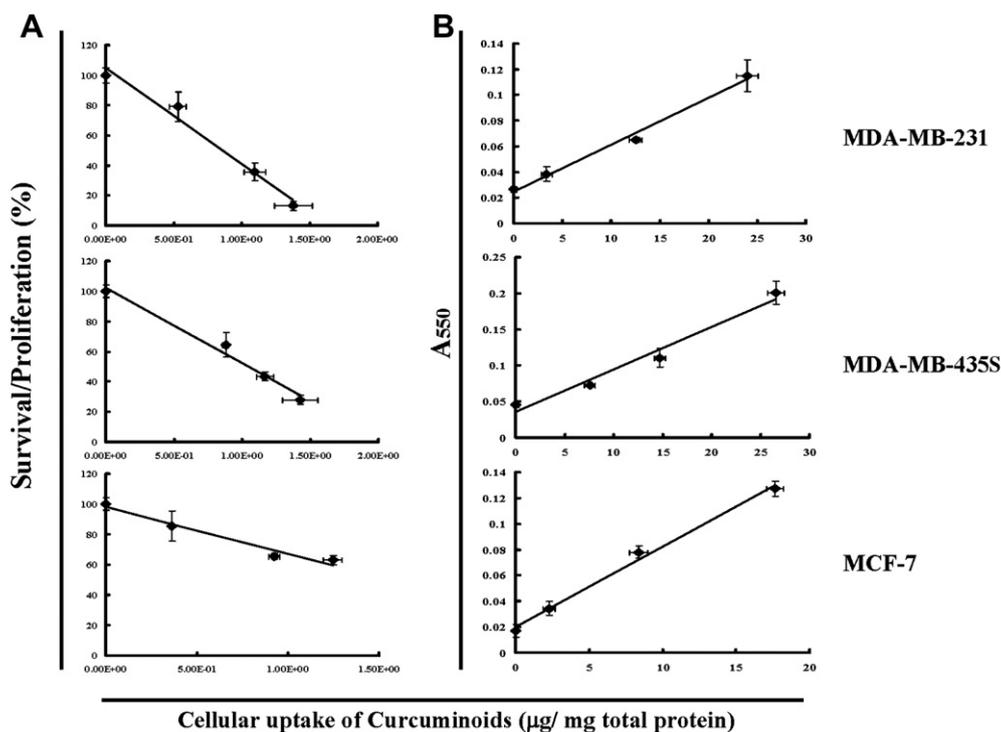


Fig. 4. Correlation between the percentage of inhibition in breast cancer cells and curcuminoid uptake. (A) A linear correlation exists between the percentage of inhibition of the proliferation of breast cancer cells (data from Fig. 1A) and the logarithmic amount of curcuminoids taken up by the cancer cells (data from Table 1). The linear correlation, which has a correction coefficient of greater than 0.982, could be described by the following equation: % of inhibition = Intercept - $P \log$ (cellular uptake of curcuminoids), where $P = -63.95$ for MDA-MB-231 ($R^2 = 0.989$), $P = -49.799$ for MDA-MB-435S ($R^2 = 0.991$), $P = -30.7$ for MCF-7 ($R^2 = 0.982$). (B) A linear correlation existed between the level (A550) of induction of apoptosis of breast cancer cells (data from Fig. 1B) and the amounts of curcuminoids taken up by the cancer cells (data from Table 1). The linear correlation, which has a correction coefficient of greater than 0.986, can be described by the following equation: $A550 = \text{Intercept} - R$ (cellular uptake of curcuminoids), where $R = 0.0036$ for MDA-MB-231 ($R^2 = 0.996$), $R = 0.0059$ for MDA-MB-435S ($R = 0.986$, $R^2 = 0.0062$ for MCF-7 ($R^2 = 0.997$).

The results summarized in Fig. 3A demonstrate that the analytical methods developed in this study for the assay of curcumin and its demethoxylated derivatives (DMC and bDMC) have several advantages: (1) the short running time (<15 minutes) required for the qualitative determination and quantitative assay of curcumin and its demethoxylated derivatives (DMC and bDMC) coexisting in the curcuminoids; (2) a simple extraction procedure providing a highly efficient recovery of curcumin and its demethoxylated derivatives from cell cultures and culture medium; and (3) assays with a high degree of sensitivity and good correlation. Moreover, the proposed method outperforms other methods reported in the literature with regard to sensitivity of detection [16–18,20]. Compared to the methodologies described previously, the HPLC method developed in this study is faster, provides greater sensitivity, accuracy, and reproducibility, and is more easily standardized.

The proposed method is capable of providing a qualitative and quantitative determination of curcuminoids in cells, tissues, and organs for a wide range of research purposes and preclinical or clinical applications in breast cancer therapy.

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