Cyclodextrin-complexed curcumin exhibits anti-inflammatory and antiproliferative activities superior to those of curcumin through higher cellular uptake

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ABSTRACT

Curcumin, a yellow pigment present in the spice turmeric (*Curcuma longa*), has been linked with multiple beneficial activities, but its optimum potential is limited by poor bioavailability, in part due to the lack of solubility in aqueous solvents. To overcome the solubility problem, we have recently developed a novel cyclodextrin complex of curcumin (CDC) and examined here this compound for anti-inflammatory and antiproliferative effects. Using the electrophoretic mobility shift assay, we found that CDC was more active than free curcumin in inhibiting TNF-induced activation of the inflammatory transcription factor NF-κB and in suppressing gene products regulated by NF-κB, including those involved in cell proliferation (cyclin D1), invasion (MMP-9), and angiogenesis (VEGF). CDC was also more active than free curcumin in inducing the death receptors DR4 and DR5. Annexin V staining, cleavage of caspase-3 and PARP, and DNA fragmentation showed that CDC was more potent than free curcumin in inducing apoptosis of leukemic cells. Antiproliferative assays also demonstrated that CDC was more active than free curcumin in suppressing proliferation of various cancer cell lines. The cyclodextrin vehicle had no effect in these assays. Compared with free curcumin, CDC had a greater cellular uptake and longer half-life in the cells. Overall we demonstrated that CDC had superior attributes compared with free curcumin for cellular uptake and for antiproliferative and anti-inflammatory activities.

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1. Introduction

Cancer is a hyperproliferative disorder that is usually treated by radiation and chemotherapeutic agents that are toxic not only to tumor cells but also to normal cells. In addition to being toxic, these agents are often not very effective in increasing overall survival of cancer patients and can be very expensive, and thus are not affordable for most. Moreover, such agents cannot be used for cancer prevention. Traditional medicines are generally free of deleterious side effects and are usually inexpensive. Curcumin (diferuloylmethane), an active component of the perennial turmeric (*Curcuma longa*), is one such agent that is safe, affordable, and efficacious [1]. Curcumin has been shown to suppress pathways linked to oncogenesis, including those involved in cell survival, proliferation, invasion, and angiogenesis [2].

Curcumin has been used as a spice and as an Ayurvedic medicine for centuries in the Indian subcontinent. This nutraceutical has been shown to suppress carcinogenesis in the skin, liver, lung, colon, stomach, and breast. Extensive research within the last two decades has shown that this agent exhibits antioxidant, anti-inflammatory, anti-survival, antiproliferative, anti-invasive, and antiangiogenic activity [3]. Curcumin also inhibits the proliferation of a wide variety of tumor cells in culture and promotes apoptosis through Bid cleavage, cytochrome *c* release, and activation of caspase-9 and caspase-3 [4]. These effects are mediated in part through the downregulation of various transcription factors, including nuclear factor (NF)-κB [5,6], activator protein (AP)-1 [7], hypoxia inducible factor (HIF)-1α [8], and beta-catenin [9]. The downregulation of these factors in turn leads to the down-regulation of various proteins involved in cell proliferation (e.g., cyclin D1), invasion (e.g., matrix metalloproteinase-9 [MMP-9]) and angiogenesis (e.g., vascular endothelial growth factor [VEGF]).

Various animal studies have shown that curcumin can prevent carcinogen-induced tumorigenesis and also can inhibit implanted human tumors in rodents [10,11]. Such studies have been followed by clinical trials of curcumin in patients with pancreatic cancer [12], colon cancer [13,14], and multiple myeloma [15].

Despite the promising biological effects of curcumin, its poor oral bioavailability in both rodents and humans [16,17] has restricted its use in the management of human ailments. It is well known that many drugs have bioavailability problems due to their low water solubility, slow dissolution rate, and instability in the gastrointestinal tract. Poor oral absorption of curcumin due to its extremely low aqueous solubility or extensive presystemic metabolism may be responsible for the unfavourable pharmacokinetics of this molecule [18]. Various methods have been tried to enhance curcumin delivery, including its incorporation into liposomes [19,20], phospholipid vesicles [21], and nanoparticles [22].

To overcome this solubility problem, we have recently developed a novel cyclodextrin complex of curcumin (CDC). Cyclodextrins are cyclic oligosaccharides with a hydrophilic outer surface and lipophilic central cavity. Hydrophilic drug/cyclodextrin complexes are formed by the inclusion of lipophilic drugs or lipophilic drug moieties in the central cyclodextrin cavity. Cyclodextrins have been frequently used as solubilizing and stabilizing agents in pharmaceutical preparations [23]. Earlier attempts to manufacture pharmaceutically acceptable cyclodextrin complexes for curcumin have been hampered by limited solubility of curcumin, high molar excess of cyclodextrins required and impaired photostability [24]. However, these complexes had promising bioavailability in vivo [25]. The novel cyclodextrin complex is manufactured by a pH shift method using a highly alkaline solution for curcumin dissolution and contacting with hydroxypropyl-γ-cyclodextrin (HPγCD). After neutralization of the solution, a stable complex of curcumin with HPγCD is recovered (L. Vaahtera, A. Hietala, V. Aseyev, H. Tenhu, J. Parkkinen, unpublished results).

In this study, we have further characterized this novel curcumin formulation and assessed it for various cellular responses and for cellular uptake.

2. Materials and methods

2.1. Reagents

We got curcumin (Curcumin C3 Complex) as a gift sample from Sabinsa, East Windsor, NJ. 10 mM stock solution of curcumin in DMSO was prepared and stored at −20 °C. For further use, it was diluted with culture medium to get desired concentration of curcumin and also to keep the final DMSO concentration less than 0.1%. 2-Hydroxypropyl-γ-cyclodextrin was purchased as Cavasol W8 Pharma from Wacker Chemie (Munich, Germany). Ammonium persulphate, ferrous ammonium sulphate, and N,N'-methylene bis-acrylamide were obtained from Sigma Chemical (St. Louis, MO). Tetramethylethylenediamine (TEMED) was purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum was supplied by Atlanta Biologicals (Lawrenceville, GA). Antibodies against cyclin D1, MMP-9, caspase-3, PARP and death receptors DR-4 and DR-5 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Bacteria-derived human recombinant tumor necrosis factor (TNF), purified to homogeneity with a specific activity of 5×10^7 U/mg, was provided by Genentech (South San Francisco, CA). Penicillin, streptomycin, Iscove's modified Dulbecco's medium, Dulbecco's modified Eagle's medium, RPMI medium, and the Live/Dead viability/cytotoxicity kit were obtained from Invitrogen (Grand Island, NY). An anti-VEGF antibody was purchased from Thermo Scientific (Fremont, CA).

2.2. Cell lines

The cell lines KBM-5 (human chronic myeloid leukemia), SCC-4 (human head and neck squamous cancer), Caco-2 (human colonic carcinoma), and Panc-28 (pancreatic cancer) were obtained from American Type Culture Collection (ATCC, Manassas, VA). KBM-5 cells were cultured in Iscove's modified Dulbecco's medium with 15% fetal bovine serum; Caco-2, and Panc-28 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. SCC-4 cells were cultured in DMEM supplemented with 10% FBS, 100 μM nonessential amino acids, 1 mM Pyruvate, 6 mM L-glutamine and 1X vitamins. Culture media were supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin.

2.3. Formulation of the cyclodextrin complex of curcumin

Hydroxypropyl-γ-cyclodextrin was dissolved to a concentration of 112 g/L in 0.18 mol/L sodium hydroxide solution. Curcumin (Curcumin C3 Complex; Sabinsa Corporation, East Windsor, NJ) was added to a concentration of 15 g/L. The solution was agitated and after complete dissolution of curcumin, the pH was adjusted to 6.0 with a mixture of hydrochloric acid and citric acid. The solution was sterile filtered and filled aseptically into sterile vials, capped and sealed. The recovered CDC solution contained 12 g/L curcumin and 93 g/L cyclodextrin in 20 mM sodium citrate, 100 mM NaCl solution. Endotoxin content was less than 1.8 IU/mL as measured by the Limulus amebocyte lysate gel clot method. The CDC solution was stored at 2–8 °C protected from light. The cyclodextrin vehicle was prepared in the same way but without the addition of curcumin.

2.4. Electrophoretic mobility shift assay

To assess NF-κB activation, we isolated nuclei from cells and performed electrophoretic mobility shift assays (EMSAs) essentially as previously described [26]. In brief, nuclear extracts prepared from cancer cells (2×10^6) were incubated with ^{32}P-end-labeled 45-mer double-stranded NF-κB oligonucleotide (4 μg of protein with 16 fmol of DNA) from the HIV long terminal repeat (5′-TTGTTACAA**GGGACTTTC** CGCTG **GGGACTTTC** CAGGGA GGCGT GG-3′; boldface indicates NF-κB binding sites) for 15 min at 37 °C. The resulting DNA-protein complex was separated from free oligonucleotides on 6.6% native polyacrylamide gels. A double-stranded mutant oligonucleotide (5′-TTGTTACAA**CTCACTTTC** CGCTG**CTCACTTTC** CAGGGAGG CGTGG-3′) was used to evaluate the specificity of binding of NF-κB to DNA. The dried gels were visualized, and radioactive bands were quantified using a Phosphorimager imaging device (Molecular Dynamics, Sunnyvale, CA) and ImageQuant software.

2.5. Immunocytochemical analysis for NF-κB p65 localization

The effect of CDC on TNF-induced nuclear translocation of p65 was examined after 4 h of treatment using an immunocytochemical method. Slides were analyzed under a fluorescence microscope (Labophot-2; Nikon, Melville, NY), and images were captured using a Photometrics Coolsnap CF color camera (Nikon, Melville, NY) as described previously and acquired with MetaMorph 4.6.5 software (Universal Imaging, PA) [27].

2.6. Western blot analysis

Cells were incubated on ice for 30 min in 0.5 mL of ice-cold whole-cell lysate buffer (10% NP-40, 5 mM NaCl, 1 mM HEPES, 0.1 mM EGTA, 0.5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium orthovanadate, 1 mM NaF, 2 μg/mL aprotinin, and 2 μg/mL leupeptin). Proteins were then fractionated by

SDS-polyacrylamide gel electrophoresis, electrotransferred to nitrocellulose membranes, blotted with each antibody, and detected by enhanced chemiluminescence (GE Healthcare, NJ). Densitometric quantitation was determined by Image Quant 5.1 software.

2.7. Cell proliferation assay

The cytotoxicity effects of cyclodextrin (CD), curcumin and CDC were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) uptake method. Briefly, 50,000 cells were incubated with curcumin and CDC in triplicate in a 96-well plate for 72 h at 37 °C. An MTT solution was added to each well, and the cells were incubated for 2 h at 37 °C. An extraction buffer (20% SDS and 50% dimethylformamide) was added, and the cells were incubated overnight at 37 °C. The absorbance was then measured at 570 nm using a 96-well multi-scanner (MRX Revelation; Dynex Technologies, VA).

2.8. Cell viability assay

We used Live/Dead assay kit (Molecular Probes, Eugene, OR), which is based on staining of viable cells with green fluorescent calcein and dead cells with red fluorescent ethidium bromide. In brief, cells (5000 per well) were incubated with curcumin and CDC for 24 h. Cells were then stained with assay reagents for 30 min at ambient temperature. Cell viability was determined under a fluorescence microscope by counting live (green) and dead (red) cells.

2.9. Apoptosis assays

Cells were pretreated with CDC, curcumin or the cyclodextrin vehicle (CD) for 24 h at different concentrations, and analysed for various parameters of apoptosis. An early indicator of apoptosis is the rapid translocation and accumulation of the membrane phospholipid phosphatidylserine from the cytoplasmic interface of membrane to the extracellular surface. This loss of membrane asymmetry can be detected by annexin V staining. For this, the cells were washed in PBS, resuspended in 100 μL of binding buffer containing a FITC conjugated anti-annexin V antibody, and then analyzed with a flow cytometer (FACS Calibur, Becton Dickinson, Bedford, MA) as described elsewhere [28]. A total of 10,000 events were analyzed by flow cytometry using an excitation wavelength at 488 nm and emission 610 nm.

For the analysis of caspase-3 and PARP cleavage, the treated cells were lysed, and processed for western blot analysis using anti-caspase-3, and anti-PARP antibodies, respectively. Propidium iodide (PI) staining for analysis of DNA fragmentation was performed by FACS analysis (FACS Calibur, Becton Dickinson, Bedford, MA) as described elsewhere [28].

2.10. Curcumin and CDC uptake in cells

The cellular uptakes of curcumin and CDC in KBM-5 cells were analyzed by the fluorescence method. In brief, cells (1×10^6) were incubated with curcumin or CDC for different time intervals. Vehicle controls were kept for each of the treatment condition. We also used Hoechst's reagent for staining the nucleus. Cells were then examined under a fluorescence microscope (Labophot-2; Nikon, Melville, NY), and images were captured using a Photometrics Coolsnap CF color camera (Nikon, Melville, NY). The emission spectra were recorded from 450 to 700 nm with an excitation wavelength at 425 nm for curcumin and CDC. At least three monochrome images were accumulated from three different microscopic fields of the same slide. The desired region on each fluorescent cell was selected and the mean fluorescence intensity/

area for the region was determined using the Median Fluorescence Intensity version 3.4kβ8 and the average values were presented.

For quantification of cellular uptake, a method was adopted as described earlier by Kunwar et al. [29]. KBM-5, SCC-4, Caco-2, and Panc-28 cells were suspended at 5×10^6 cells/mL using serum free medium with desired concentration of curcumin and CDC in 12 well plates. The concentration of curcumin is expressed as pmol/million cells/mL. Vehicle controls were kept for each of the treatment condition. At different incubation period, cells were pelleted at 1000 rpm in Eppendrof centrifuge ($200 \times g$) for 5 min and washed twice with cold phosphate buffered saline (PBS). The pellet was dried and suspended in 1 mL of methanol and sonicated for 5 min, so that curcumin was extracted into the methanol fraction. This methanol fraction was further centrifuged at 10,000 rpm for 5 min and absorption spectrum of supernatant containing methanolic curcumin was recorded at 425 nm, the amount of curcumin loaded to cells was estimated. Curcumin in the lysate was also determined by HPLC (Waters separation module Alliance 2695, and dual λ UV detector 2487; Waters Corporation, Milford, MA). Silica-based C18 column and mobile phase that was a mixture of acetonitrile:tetrahydrofuran:water (containing 1% citric acid) in a ratio of 12:25:63, respectively, were used for separation. An uptake was expressed as pmol of curcumin or CDC per million cells/mL. Mean and SD were calculated from three independent experiments.

2.11. Statistical analysis

For statistical calculations, the replicate data from cell proliferation experiments, EMSA, densitometric analysis of western blots, fluorescence intensity and HPLC analysis were compared for the quantitative differences by the non-parametric Wilcoxon test.

A P value of less than 0.05 was considered statistically significant. Statistical evaluations were performed with the SAS version 9.2 (SAS institute, Cary, NC). In all graphs, error bars represent SD.

3. Results

The novel cyclodextrin complex of curcumin (CDC) manufactured by the pH shift method differentiated in several ways from the previously described cyclodextrin complexes of curcumin. When we tested the dissolution of curcumin in 10% HPγCD solution at a slightly acidic pH, only 0.5 mM solution of curcumin was obtained in the presence of a high molar excess of cyclodextrins, similar to as described earlier [24]. Using the pH shift method, we could manufacture curcumin solutions, which contained >50 mM curcumin in the presence of a slight molar excess of HPγCD. A sterile solution of the CDC complex has remained remarkably stable during long term storage, for more than 2 years by now at 2–8 °C.

CDC was compared with free curcumin dissolved in DMSO for its ability to suppress TNF-induced NF-κB activation and NF-κB-regulated gene products, to induce apoptosis, and to suppress proliferation of tumor cells. We also investigated the cellular uptake and the half-life of intracellular curcumin. Because the effects of curcumin on human leukemia KBM-5 cells have been well documented [30], these cells were extensively used in this study, but the results were confirmed with three other cancer cell lines also.

3.1. CDC is more potent than free curcumin for inhibition of NF-κB activation

Suppression of NF-κB is one of the major activities of curcumin. We therefore compared the effects of CDC and free curcumin in
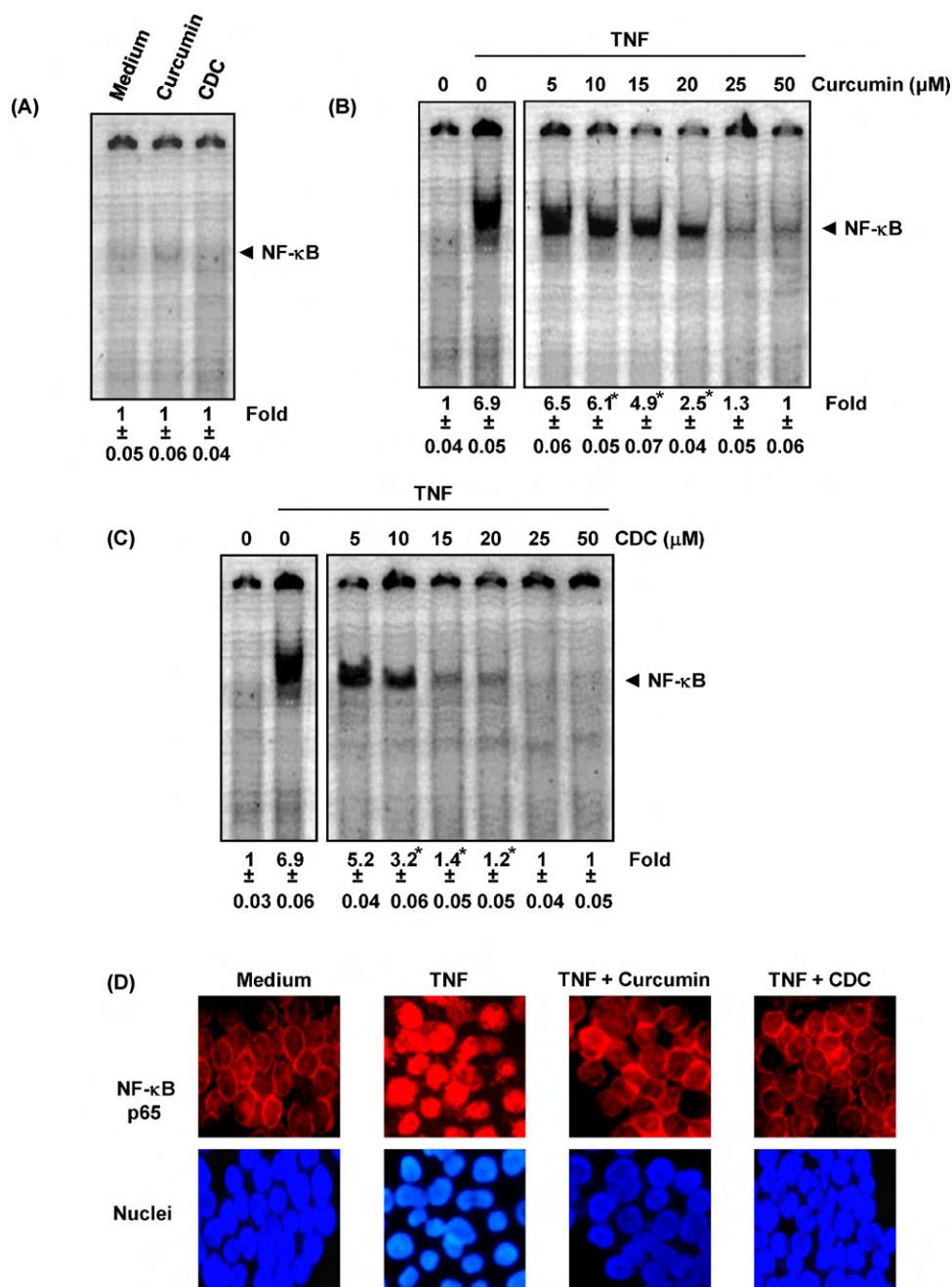
Fig. 1. (A) CDC does not induce NF-κB activation in KBM-5 cells. KBM-5 cells (2×10^6) were treated with the 50 μM of curcumin or CDC for 4 h. Nuclear extracts were prepared and the NF-κB activity was examined by EMSA. (B and C) CDC is more potent than regular curcumin in inhibiting TNF-induced activation of NF-κB. KBM-5 cells (2×10^6) were treated with the indicated concentrations of curcumin (B) or CDC (C) for 4 h. The cells were then incubated with 0.1 nM TNF for 30 min and analyzed for NF-κB activity by EMSA. The figures shown are representative of three independent experiments, and the numerical values expressed as mean ± SD are calculated from three independent experiments. (D) Immunocytochemical analysis of TNF-induced p65 nuclear translocation. KBM-5 cells were incubated with 10 μM curcumin or 10 μM CDC for 4 h, treated with 0.1 nM TNF for 30 min, and then subjected to immunocytochemical analysis and examined in a fluorescence microscope. The figures shown are representative of three independent experiments. *$P < 0.05$.

suppressing TNF-induced NF-κB activation. KBM-5 cells were treated with 10–50 μM of curcumin or CDC for 4 h, and nuclear extracts were prepared and analyzed for NF-κB activity by EMSA. The figures are the representative of one of the three independent experiments. The numerical values below the figure represent the means and standard deviation calculated from three independent experiments. Neither curcumin alone nor CDC alone activated NF-κB (Fig. 1A). Free curcumin and CDC, both inhibited the TNF-induced activation of NF-κB, in a dose-dependent manner (Fig. 1B and C). CDC was more potent than free curcumin in inhibiting TNF-induced NF-κB activation. At the dose of 5–25 μM CDC suppressed

significantly NF-κB induction than curcumin ($P < 0.05$). Under these conditions, neither curcumin nor CDC had any significant effect on cell viability, indicating that suppression of NF-κB activation was not due to loss of cell viability.

3.2. CDC inhibits nuclear translocation of NF-κB p65

Because IκBα degradation is required for nuclear translocation of p65, we sought to determine whether CDC also suppresses TNF-induced nuclear translocation of p65. Immunocytochemical analysis showed that CDC suppressed the TNF-induced transloca-
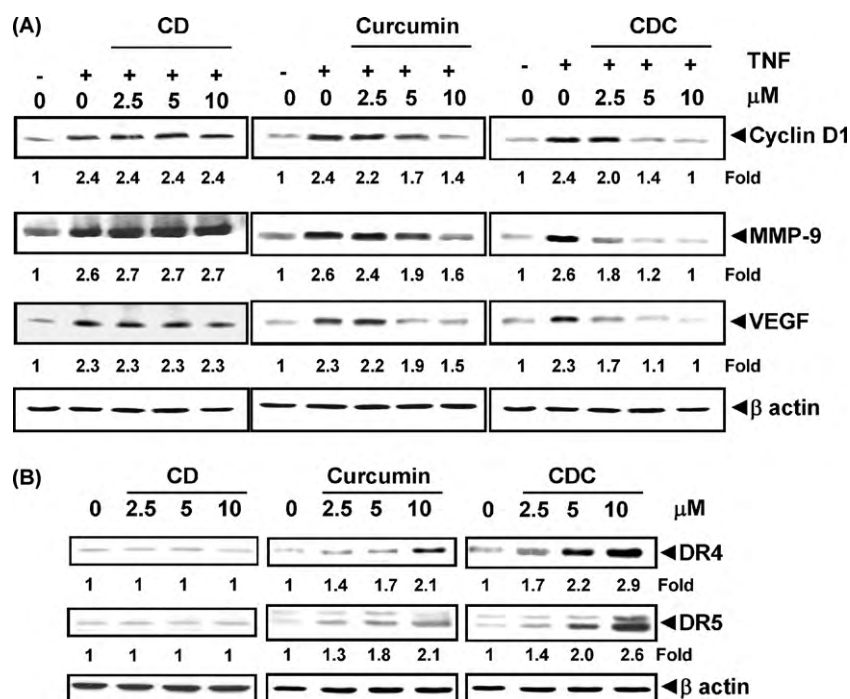
Fig. 2. CDC is more potent than curcumin in inhibiting TNF-induced expression of NF-κB-regulated genes and death receptors. KBM-5 cells (1 × 10⁶) were co-incubated with TNF (1 nM) and the indicated concentrations of curcumin or CDC for 24 h. The cells were harvested, and the expressions of cyclin D1, MMP-9, VEGF, and death receptors (DR4 and DR5) were analyzed by western blot. β-Actin was used as a loading control. The figures shown are representative of three independent experiments.

tion of p65 to the nucleus in KBM-5 cells (Fig. 1D). In both untreated cells and cells treated with CDC or curcumin, p65 was localized in the cytoplasm, but in cells treated with TNF, p65 was translocated to the nucleus. These results confirmed that CDC inhibited translocation of p65.

3.3. CDC effectively downregulates the expression of NF-κB-regulated gene products

We compared the ability of CDC, curcumin and the cyclodextrin vehicle (CD) to downregulate the expression of NF-κB-regulated gene products associated with proliferation, survival, invasion, and angiogenesis. KBM-5 cells were co-incubated with TNF and various concentrations of CDC, curcumin and CD; and then examined for cyclin D1 (cell proliferative), MMP-9 (invasion), and VEGF (angiogenesis) gene products. As shown in Fig. 2A, there was a trend for more effective suppression of all these gene products by CDC when compared to free curcumin. Notably, the cyclodextrin vehicle had no effect.

3.4. CDC effectively upregulates death receptor expression

Curcumin has been shown to upregulate the expression of death receptors, DR4 and DR5. Hence, we investigated whether CDC can also modulate these death receptors. As shown in Fig. 2B, CDC induced the expression of both DR4 and DR5 proteins in a dose-dependent manner and there was a trend for more effective induction by CDC than curcumin. Again, the cyclodextrin vehicle had no effect.

3.5. CDC is more potent than free curcumin for suppression of tumor cell proliferation

Next we determined whether CDC inhibits the proliferation of KBM-5 (human chronic myeloid leukemia), SCC-4 (human head and neck squamous cancer), Caco-2 (human colonic carcinoma), or
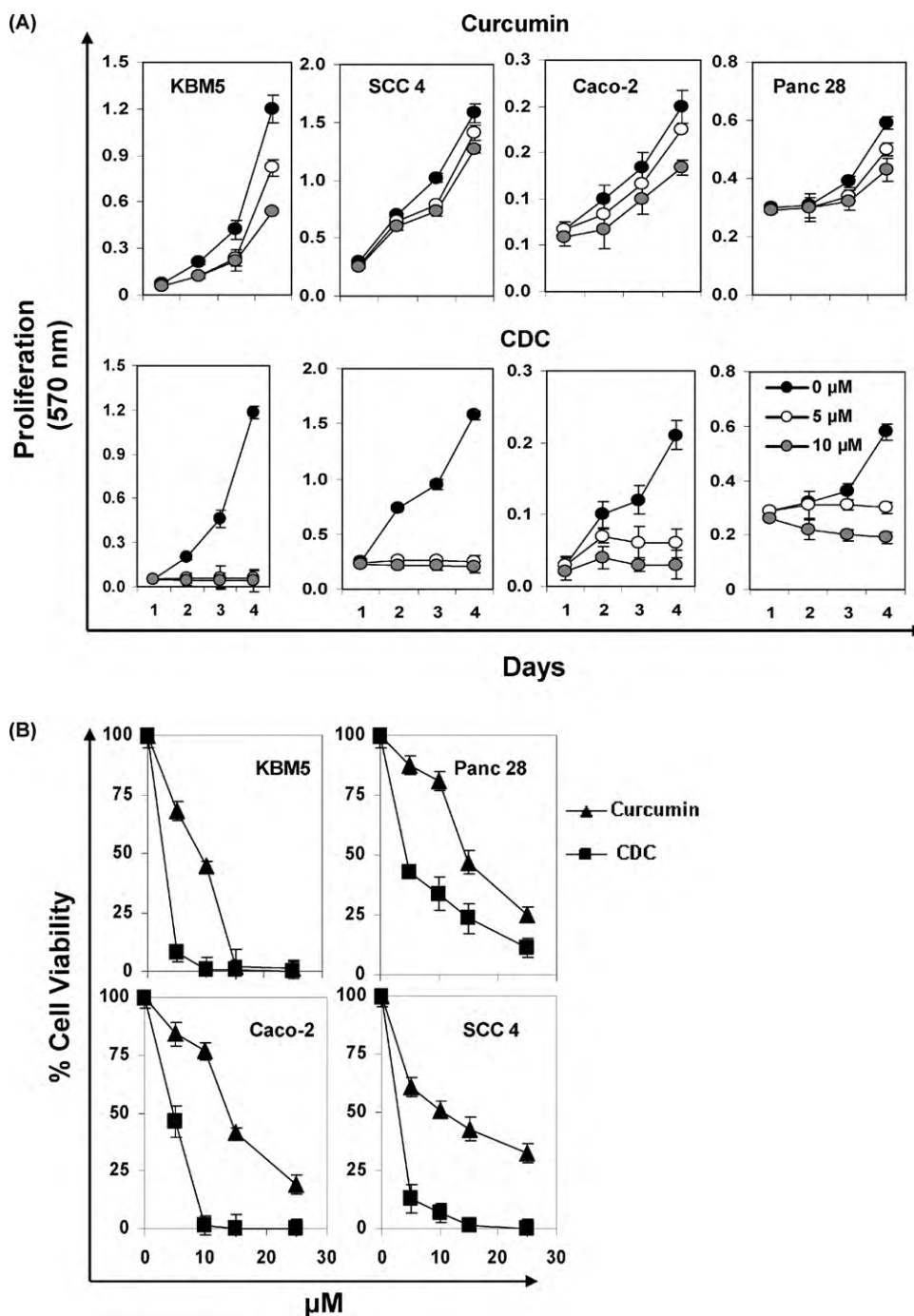
Panc-28 cells (pancreatic cancer). Cells were incubated with different concentrations of either curcumin or CDC for 72 h and then examined for apoptosis by the MTT method. CDC inhibited cell proliferation in all four cell lines in a dose-dependent manner and statistically more effectively than free curcumin (Fig. 3A and B).

3.6. CDC is more potent than free curcumin for induction of tumor cell apoptosis

We first evaluated the ability of CDC to induce apoptosis in human cancer cells using the Live/Dead apoptosis assay. When human KBM-5 cells were incubated with different concentrations of CDC or curcumin for 24 h, ethidium bromide staining showed a dose-related loss of cell viability which was more pronounced with CDC than curcumin (Fig. 4A). We also examined the effect of curcumin and CDC on the activation of caspase-3, and poly (ADP-ribose) polymerase (PARP) cleavage. We found that curcumin and CDC cleaved the caspase-3, and PARP in dose-dependent manner (Fig. 4B).

We next determined whether CDC, curcumin and the cyclo-dextrin vehicle induced DNA degradation, a hallmark of apoptosis, in KBM-5 cells. DNA analysis by flow cytometry was performed after 24 h incubations of KBM-5 cells within increasing concentrations of CDC, curcumin and the cyclodextrin vehicle. The free CD did not show any DNA degradation, while curcumin and CDC induced DNA degradation in a dose-dependent manner (Fig. 4C and Table 1). After exposure to 25 μM curcumin, 15.1% DNA was fragmented while same concentration of CDC induced nearly twofold more DNA fragments (28.2%).

We next determined whether CDC, curcumin and CD induced apoptosis in KBM-5 cells by annexin V staining, which detects an early stage of apoptosis, and combined staining with propidium iodine, which detects a late stage of apoptosis. These results indicated that CD did not induced apoptosis, while curcumin and CDC showed apoptosis in a dose-dependent manner. The propor-

Fig. 3. (A) Effect of CDC and curcumin on proliferation/survival as assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. CDC is more active than curcumin in suppressing proliferation at different days. (B) CDC showed more cytotoxic in different cancer cells. KBM-5 (human chronic myeloid leukemia), SCC-4 (human head and neck squamous cancer), Caco-2 (human colonic carcinoma), and Panc-28 cells (pancreatic cancer) were incubated with different concentrations of either curcumin or CDC in for 72 h and then examined for cell viability by the MTT method. The results show the mean ± SD values.

tion of annexin V positive cells after 24 h treatment was 49.9% in case of curcumin and 77.2% in case of CDC (Fig. 4D and Table 1). Taken together, all these results suggest that CDC showed more apoptosis than free curcumin.

3.7. Cellular uptake of curcumin from CDC is higher than the uptake as free curcumin

Cellular uptake as a function of curcumin treatment at different time intervals was estimated by fluorescence microscopy. Fig. 5A shows the kinetics of uptake of CDC and curcumin in KBM-5 cells,

after treatment with 10 μM CDC or 10 μM curcumin. The results indicated that the uptake increased with increasing time intervals. Cell viability was confirmed by blue color in Hoechst's staining.

The cellular uptake of curcumin from CDC and as free curcumin by different cells was quantified by separating of curcumin from cell lysates by HPLC (Fig. 7A and B). The average uptake of curcumin calculated from the absorption spectra, expressed as pmol/million cells for different cell lines are given in Table 2. The results indicate that the uptake of curcumin from CDC continued somewhat longer, about 60 min, than as for free curcumin, which peaked at 45 min. The total amount of curcumin taken up from the
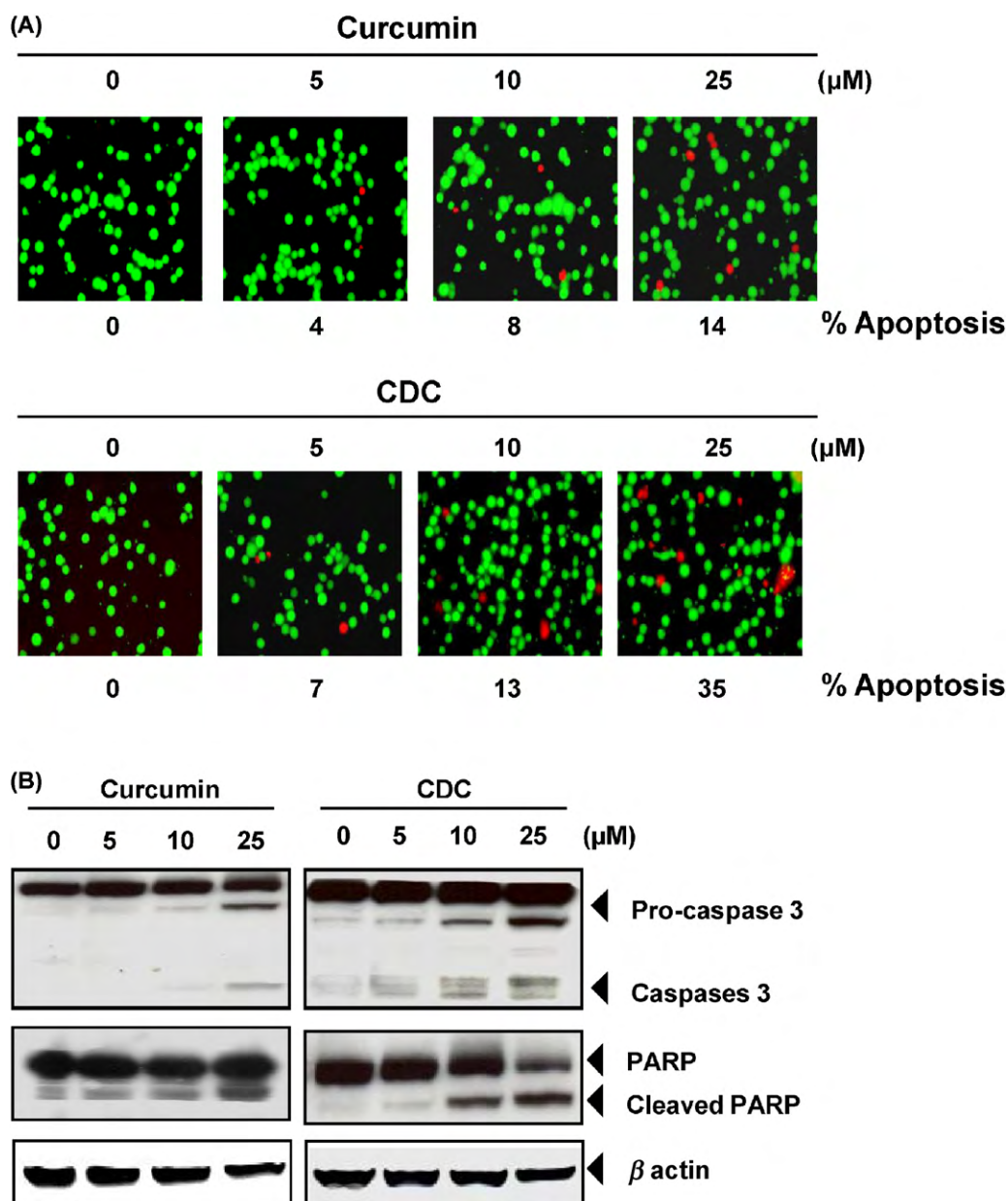
Fig. 4. CDC is more active than curcumin in inducing apoptosis. (A) KBM-5 cells (50,000 cells/well) were incubated with curcumin or CDC at the indicated concentrations for 24 h. The cells were harvested and stained with Live/Dead assay reagent as per the manufacturer's protocol as described in Section 2. The results shown are representative of three independent experiments. (B) CDC showed more apoptosis than curcumin. KBM-5 cells were treated with (5–25 μM) CD, curcumin or CDC for 24 h. Cleavage of caspase-3, and poly (ADP-ribose) polymerase was determined by western blotting in whole-cell extracts of CD, curcumin, and CDC treated cells. The results shown are representative of three independent experiments. (C) CDC showed more cell death than curcumin. KBM-5 cells (2×10^6/mL) were synchronized by incubation overnight in the absence of serum and then treated with (5–25 μM) CD, curcumin and CDC for 24 h, after which the cells were washed, fixed, stained with propidiumiodine and analyzed for DNA content by flow cytometry. (D) KBM-5 cells were treated with (5–25 μM) CD, curcumin and CDC for 24 h. Cell death was determined by fluorescence-activated cell sorting using annexin V/propidium iodide staining. (Fig. 4. *continued*)

CDC was correspondingly higher. Interestingly, the rate of disappearance of curcumin from the cells was found to be slower with CDC than with free curcumin.

3.8. Cellular release of CDC is slower than free curcumin

We further investigated the half-life of curcumin in the cells after removing the curcumin formulations from the medium. For this, cells were incubated with 10 μM curcumin or CDC for 45 and 60 min, respectively. Once uptake reached maximum, cells were washed two times with PBS and incubated for the indicated times at 37 °C. The cells were harvested at different time points, and cellular curcumin was determined by fluorescence microscopy and

HPLC. Interestingly, we found that free curcumin quickly disappeared, whereas curcumin taken up from CDC disappeared more slowly and remained longer in cells (Figs. 6 and 7A and B). Other cells showed similar results as the KBM-5 cells. The average cellular levels of curcumin, expressed as pmol/million cells for different cell lines are given in Table 3. Thus, CDC treatment resulted in more extensive and prolonged exposure of the cells to curcumin as compared to free curcumin.

4. Discussion

In the present paper, we have characterized the bioactivity of a novel cyclodextrin complex of curcumin (CDC), which is manu-
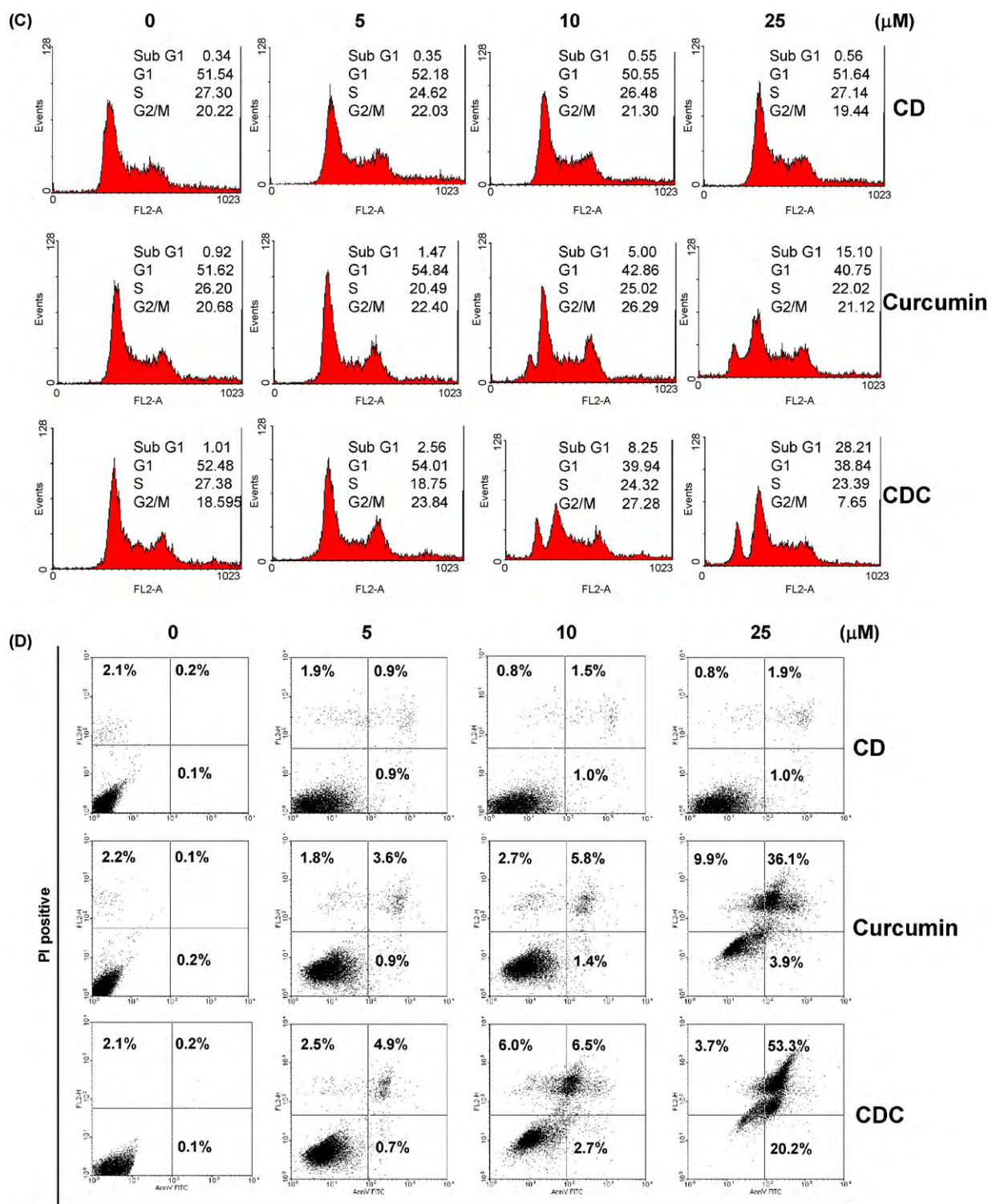
Fig. 4. (*Continuation*).

factured by a pH shift method. This method allows the manufacturing of aqueous curcumin formulations that have about 100-fold higher curcumin concentration than previously described cyclodextrin complexes of curcumin in the presence of only a slight molar excess of cyclodextrin. Furthermore, the stability of curcumin is remarkably improved in CDC, and it has remained stable as a liquid formulation for 2 years.

Our results show that CDC was more potent than free curcumin in several cellular activities, which are characteristic for the antitumor effects of curcumin. First, CDC more effectively inhibited TNF-induced NF-κB activation and suppressed NF-κB-regulated gene products involved in proliferation (cyclin D1), invasion (MMP-9), and angiogenesis (VEGF). CDC also effectively induced the expression of death receptors DR4 and DR5. Second, CDC inhibited the proliferation of several leukemia and cancer cell lines more effectively than free curcumin, and third, it induced significantly more apoptosis in these cell lines than free curcumin.

Table 1
Cell cycle arrest and apoptotic effect of CD, curcumin and CDC in KBM-5 cells.

Concentration (μM)	Cell death (%)			Apoptosis (%)		
	CD	Curcumin	CDC	CD	Curcumin	CDC
0	0.34	0.92	1.01	2.5	2.5	2.4
5	0.35	1.47	2.56	2.7	6.3	8.1
10	0.55	5.00	8.25	2.3	9.9	15.2
25	0.56	15.10	28.51	2.7	49.9	77.2

The more potent biological activities of CDC can be explained by the observed more efficient cellular uptake of curcumin from CDC than from corresponding concentrations of free curcumin. Interestingly, curcumin taken up from CDC also remained longer in the cells, leading to prolonged exposure of the cells to intracellular curcumin. It has been shown for several other hydrophobic drugs that cyclodextrins enhance their cellular uptake [23]. It remains currently unclear whether the longer half-life of intracellular curcumin taken up from CDC was due to the higher peak concentration and possibly saturated metabolism or due to different intracellular localization.

The high curcumin content of the CDC formulation should allow administration of effective doses of curcumin through different routes of administration. The potential adverse effects of cyclodextrins are based on their interactions with endogenous lipids [31]. Hydroxypropyl-γ-cyclodextrin has little if any interactions with endogenous lipids [32] and is considered safe [33]. Several drugs formulated with hydroxypropyl-β-cyclodextrin, hydroxypropyl-γ-cyclodextrin and sulfobutyl ether-β-cyclodextrin have been licensed for clinical use, including intravascular formulation of itraconazole [34]. This itraconazol formulation contains much higher amount of cyclodextrin than the CDC formulation used in the current study, which contained only 1.7-fold molar excess of cyclodextrin. This exceptionally low cyclodextrin excess reflects the stable complex formed by the pH shift method and further reduces the risk of cyclodextrin-related adverse effects.

The curcumin from CDC is released in to cells to a therapeutically active concentration, possibly by "dilution effect", which is in agreement with the general concept, where dilution is the main driving force for drug liberation from cyclodextrin complexes [35]. Cyclodextrins are generally considered to facilitate the delivery of hydrophobic drugs locally to mucosal surfaces and systemically by
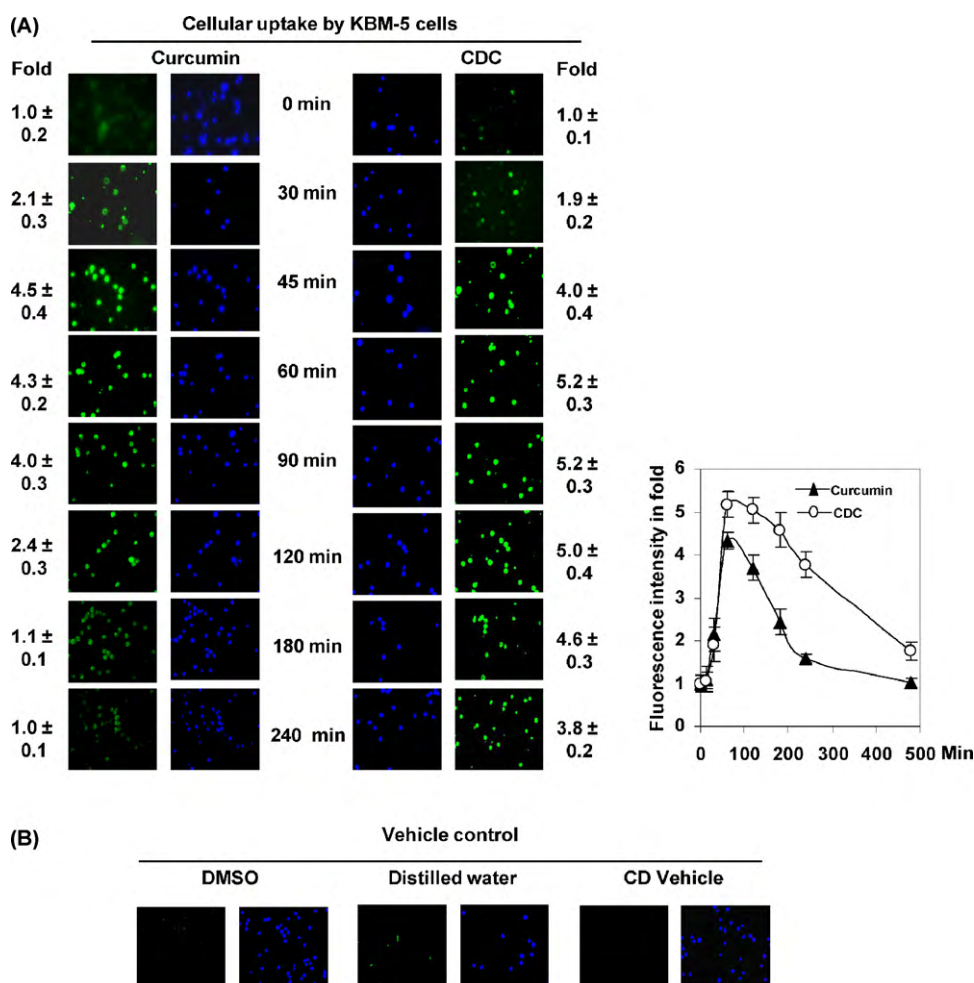
Fig. 5. (A) Cellular uptake of curcumin and CDC. KBM-5 cells (1×10^6) were incubated with curcumin or CDC at concentrations of 10 μM. The cells were harvested at the indicated times, and the cellular uptake was determined by fluorescence microscopy as described in Section 2 and blue color Hoechst's staining showed cell viability. The results shown are representative of three independent experiments. (B) The control KBM-5 cells (2×10^6) treated with DMSO and water. The results shown are representative of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Cellular uptake of curcumin and CDC in different cells.

Cell lines	Average cellular uptake (pmol/million cells) at different time (min) MV ± SD													
	30		45		60		90		120		180		240	
	C	CDC	C	CDC	C	CDC	C	CDC	C	CDC	C	CDC	C	CDC
KBM-5	22.5 ± 4.8	18.2 ± 2.2	55.3 ± 4.3*	49.9 ± 5.2	49.6 ± 5.3	69.2 ± 6.2*	46.1 ± 6.2	70.6 ± 4.4	25.7 ± 4.2	66.4 ± 5.3	12.6 ± 3.2	56.4 ± 6.6	ND	42.1 ± 7.2
SSC-4	24.4 ± 3.2	19.8 ± 3.3	57.6 ± 5.2*	50.2 ± 6.2	50.4 ± 4.8	71.2 ± 5.9*	47.1 ± 7.2	72.2 ± 5.6	27.6 ± 3.9	67.8 ± 6.3	13.8 ± 4.2	58.6 ± 5.9	ND	44.1 ± 6.3
Caco-2	20.9 ± 2.2	18.2 ± 4.4	51.3 ± 4.9*	46.7 ± 6.3	47.1 ± 5.9	66.2 ± 4.4*	42.8 ± 4.8	68.2 ± 4.9	23.9 ± 4.1	64.8 ± 2.2	12.8 ± 2.2	53.6 ± 4.9	ND	38.3 ± 4.2
Panc-28	23.7 ± 4.1	19.1 ± 2.2	52.7 ± 3.9*	42.9 ± 5.3	48.3 ± 4.4	68.3 ± 4.7*	43.1 ± 6.6	69.2 ± 4.7	25.2 ± 5.5	66.2 ± 4.9	12.9 ± 2.2	56.8 ± 4.4	ND	42.7 ± 2.9

ND, not detected (below the estimation level); MV, mean value; SD, standard deviation.
* $P < 0.05$.

oral and different parenteral routes. Preclinical studies with CDC have indicated efficient partitioning of curcumin into tissues from the CDC complex. CDC also proved safe after intravascular administration to dogs in a dose escalation study (J. Parkkinen and G. Georges, unpublished results). Our results are also in agreement with previous studies that have demonstrated that cyclodextrin-complexed curcumin exhibits enhanced anti-inflammatory and antiangiogenic activities in the inflammatory bowel disease (IBD)-induced rat model [25]. Cyclodextrin-complexed curcumin has also been functionalized with folic acid for folate receptor-overexpressing tumors [36]. Another study showed that for radical scavenging activity, curcumin is more active than the cyclodextrin-complexed curcumin [37]. This suggests that the free phenolic hydroxyl group may be essential for the scavenging properties and that the two halves of the symmetric curcumin molecule act as two separate units and scavenges one radical each. These results also suggest that the anti-inflammatory and antiproliferative activities described are independent of the radical scavenging activity of curcumin. This observation is consistent with that described by us previously [38].

Some cyclodextrins may interfere in bioactivity measurements by interfering with endogenous lipids, particularly methyl-β-cyclodextrin, which is generally used for depletion of cell membranes from cholesterol and disruption of lipid rafts [32]. Therefore, the effect of cyclodextrin vehicle was studied in parallel with CDC. The cyclodextrin vehicle did not show effect in any of the assays, which was in agreement with previous studies, in which very little, if any, interactions of hydroxypropyl-γ-cyclodextrin with endogenous lipids have been found.

The potent proapoptotic effect of CDC was demonstrated by several different methods. Caspase-3 is a key executioner of apoptosis [39], whose activation downstream in the apoptotic cascade is essential for leukemia cell apoptosis [40]. Moreover, activation of caspase-3 results in cleavage of cellular substrates critical for cell survival, such as PARP. In addition to caspase-3 activation and PARP cleavage, we used annexin V staining, which measures the loss of phospholipid asymmetry and the accumulation of phosphatidylserine to the outer leaflet of the plasma membrane taking place early during apoptosis. Finally, we measured the late phase of apoptosis by combined staining for annexin V and propidium iodine, and DNA degradation by flow cytometry, which is a definitive hallmark of apoptosis. All these assays implied that CDC may be superior to free curcumin as an antitumor agent.

The cellular uptake studies with the four different cells demonstrated that there was a preferential loading of curcumin by the cyclodextrin system as compared to free curcumin. This suggests that cyclodextrin delivery is more efficient for curcumin, and is therefore a preferred vehicle. The differential capacity of uptake of curcumin in different cell lines is probably due to different membrane structure, protein composition and size [41,42]. The enhancement of cellular uptake of drugs by cyclodextrins is based on interplay between enhanced drug solubility and decreased membrane permeability as a result of the decrease in the free fraction of drug [43]. In case of CDC, enhanced solubility of curcumin probably accounts for the favourable effect. Increased cellular uptake of curcumin from CDC could be the most important reason for its increased potency
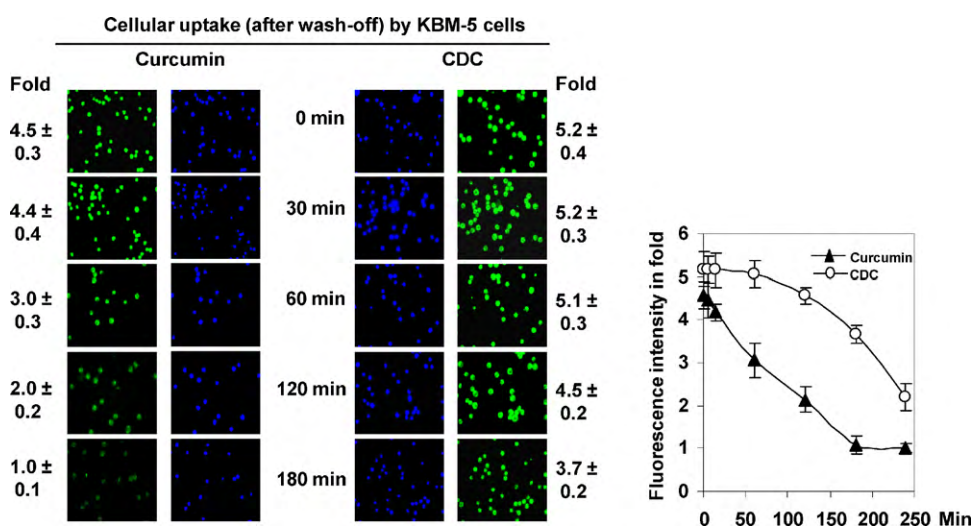
Fig. 6. Cellular release of CDC is slower than curcumin. KBM-5 cells (1×10^6) were incubated with curcumin or CDC at concentrations of 10 μM for 45 and 60 min, washed two times with PBS, and again incubated at 37 °C. The cells were harvested at the indicated times, and the cellular uptake was determined by fluorescence microscopy as described in Section 2. The results shown are representative of three independent experiments.
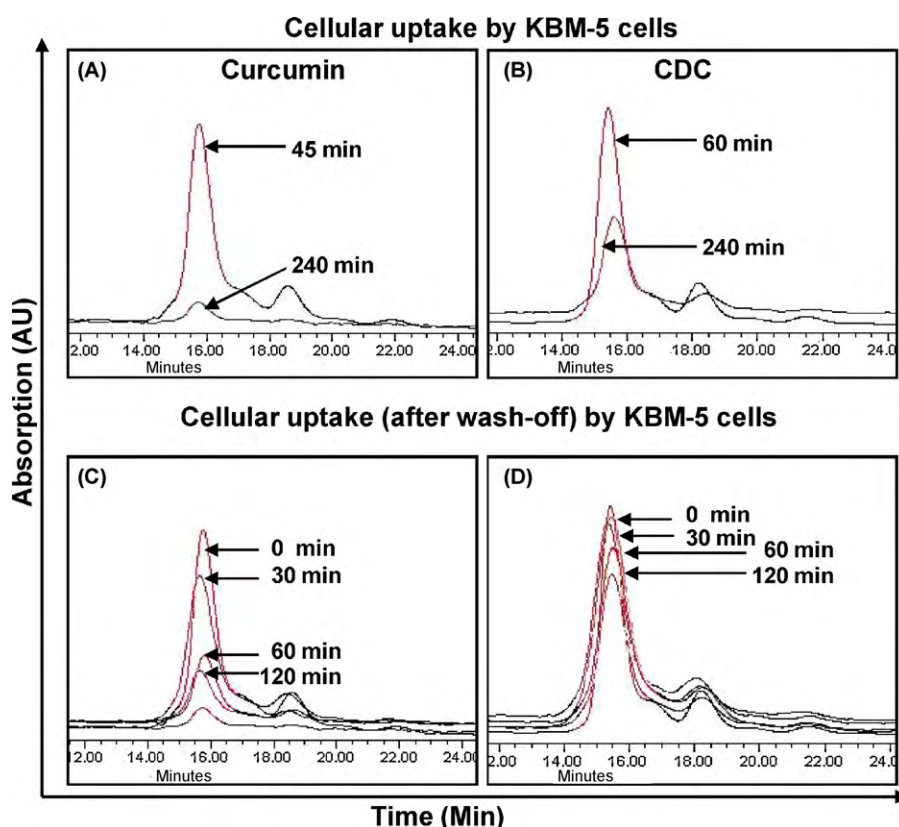
Fig. 7. Overlapped chromatograms of curcumin levels in the cells after treatment with curcumin or CDC. Chromatograms A and B show cellular uptake of curcumin and CDC, respectively, at different time points during treatment with 10 μM concentration. Chromatograms C and D show curcumin levels in the cells at different time intervals after wash off the cells. The cell density was 5×10^6/mL of KBM-5 cells.

Table 3
Cellular release of curcumin and CDC in different cells (after wash-off).

Cell lines	Average cellular uptake (pmol/million cells) at different time (min) MV ± SD									
	30		45		60		90		120	
	C	CDC	C	CDC	C	CDC	C	CDC	C	CDC
KBM-5	55.3 ± 4.3	69.2 ± 6.2	52.2 ± 2.2	69.1 ± 4.2	36.4 ± 4.3	67.2 ± 3.9	26.4 ± 4.8	59.2 ± 3.9	22.7 ± 2.9[*]	42.9 ± 4.2[*]
SSC-4	57.6 ± 5.2	71.2 ± 5.9	53.7 ± 3.7	70.8 ± 4.4	37.7 ± 2.2	67.9 ± 4.4	25.7 ± 3.7	58.7 ± 4.2	21.9 ± 3.2[*]	49.3 ± 5.2[*]
Caco-2	51.3 ± 4.9	66.2 ± 4.4	49.8 ± 3.2	68.7 ± 3.7	36.8 ± 4.4	66.7 ± 3.3	24.8 ± 3.2	56.2 ± 3.3	20.7 ± 3.6[*]	41.2 ± 4.7[*]
Panc-28	52.7 ± 3.9	68.3 ± 4.7	49.9 ± 4.4	68.4 ± 3.9	37.5 ± 3.3	64.8 ± 4.4	24.4 ± 4.4	54.6 ± 3.8	21.2 ± 3.9[*]	40.8 ± 3.3[*]

MV, mean value; SD, standard deviation.
[*] $P < 0.05$.

in inducing apoptosis in tumor cells. Being a nontoxic natural dietary product, curcumin could be useful in therapeutic strategies for cancer patients. The low molar excess of hydroxypropyl-γ-cyclodextrin needed for manufacturing of CDC and its general safety support further development of this novel formulation as an antitumor agent.

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