Modification of Curcumin with Polyethylene Glycol Enhances the Delivery of Curcumin in Preadipocytes and Its Antiadipogenic Property

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Conjugation of curcumin (CCM) by polyethylene glycol (PEG) has been previously developed to improve water solubility of the natural form of CCM and its antiproliferative role in some human cancer cell lines. This study examined the cellular uptake kinetics of the natural form of CCM and CCM−PEG. Their cytotoxic effect in proliferating preadipocytes and antiadipogenic property in differentiating preadipocytes had also been investigated. CCM and CCM−PEG were found to be differently absorbed in 3T3-L1 preadipocytes and adipocytes with a limited amount of CCM−PEG absorption in the cell. The improved water solubility of CCM−PEG was correlated with increased cellular retention of CCM in 3T3-L1 cells, particularly in preadipocytes. Consequently, CCM−PEG treatment sensitized proliferating preadipocytes to CCM-induced cell toxicity. Furthermore, incubation of differentiating 3T3-L1 cells with CCM−PEG resulted in improvement of the inhibitory role of CCM in adipocyte differentiation with no toxic effect. These results suggest that pegylation-improved water solubility and cellular retention of CCM may be uniquely useful for improving the delivery of CCM in preadipocytes and its antiadipogenic ability.

KEYWORDS: Curcumin; polyethylene glycol; solubility; bioavailability; 3T3-L1; adipogenesis

INTRODUCTION

Obesity has reached epidemic levels worldwide. Its global health hazard is closely associated with increased risks of the development of chronic diseases such as type 2 diabetes and cardiovascular disease with a rising incidence of mortality and morbidity (*1−3*). Increased adipose mass and dysfunction of adipose tissue are known to play detrimental roles in the development of obesity and its related chronic diseases. Adipogenesis, the new adipocyte generation through differentiation of preadipocytes to adipocytes, is considered to be an effective cellular target to prevent the development of obesity. Adipogenesis is tightly regulated by several transcriptional factors such as CCAAT/enhancer binding protein β (C/EBPβ), C/EBPα, and peroxisome proliferator-activated receptor γ (PPARγ) (*4−6*). Adipogenesis is also known to be inhibited by the Wnt pathway, a signal transduction pathway affecting cell growth and fate determination, which suppresses PPARγ and C/EBPα (*7*).

There has been continuous attention to identify dietary phytochemicals to prevent or delay obesity. Although a number of bioactive dietary components such as epigallocatechin gallate (*8−10*), resveratrol (*11−13*), and capsaicin (*14−16*) have been suggested to have potential antiobesity properties, the efficacy of these components under physiological conditions is largely limited by their poor bioavailability as the result of their low solubility (*17, 18*).

Curcumin (CCM, diferuloylmethane), a yellow bioactive component from rhizomes of the *Curcuma longa* plant, has a long history of dietary applications for enhancing taste and color and of medicinal use as a topical treatment for wound healing (*19*). Numerous in vitro and preclinical studies have elucidated beneficial functions of CCM as a potent preventive agent against cancer (*20−23*). This is largely through its apoptosis-inducing and cytotoxic activities in certain cancer cells at relatively high concentrations (*24, 25*). In addition, CCM also exhibits antioxidant, anti-inflammatory, and antimicrobial activities (*19, 26*). Recent in vitro and animal studies further proposed an inhibitory effect of CCM on high-fat diet-induced body weight gain and adipogenesis in adipose tissue (*27−31*). This antiadipogenic activity has been reported to be through the inhibition of various cellular and transcriptional events during the early stage of adipogenesis, including mitotic clonal expansion, Wnt signaling, and expression of proadipogenic transcription factors, C/EBPα and PPARγ (*31−33*).

Despite its safety and promising health benefits, the insolubility of dietary CCM in aqueous system limits its absorption by the gut and delivery to the bloodstream. In fact, either negligible amounts of CCM or low nanomolar quantities of CCM were found in the

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serum and liver of animals or humans after oral administration of CCM (*34*). Thus, the low water solubility of CCM is likely to limit its bioavailability. To overcome this, various approaches have been conducted, such as generation of nanoparticles (*35*), liposomes (*36*, *37*), and micelles (*38*) and encapsulation (*39*) and formulation with piperine (*40*). In addition, polyethylene glycol (PEG), a water-soluble polymer generally recognized as safe, has been used to improve the water solubility and bioavailability of drugs (*41*, *42*), dietary peptide (*43*), and dietary phytochemicals (*44*, *45*). Indeed, generation of conjugates of CCM to PEG molecules has been recently reported, and CCM−PEG has been shown to exhibit enhanced cytotoxicity in human prostate, colon, esophageal, and pancreatic cancer cells (*46*). However, the cellular uptake of water-soluble CCM−PEG in adipose tissue and its role in adipogenesis have not yet been investigated. The objective of this study is to examine the cellular uptake, cellular retention, and antiadipogenic activity of CCM−PEG in 3T3-L1 cell line, an in vitro model of adipogenesis.

MATERIALS AND METHODS

Materials and Reagents. CCM, dexamethasone (DEX), isobutylmethylxanthine (IBMX), and insulin were purchased from Sigma-Aldrich (St. Louis, MO). Fetal calf serum (FCS) and fetal bovine serum (FBS) were purchased from PAA Cell Culture Co. (Dartmouth, MA). Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, sodium pyruvate, Trizol reagent, and SuperScriptII kit were obtained from Invitrogen (Carlsbad, CA). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Alfa Aesar (Ward Hill, MA).

Synthesis of CCM−PEG. CCM−PEG generated by Safavy et al. (*46*) was employed in this study. Briefly, hydrophilic and biocompatible polymer PEG with a molecular mass of 750 Da was used for the synthesis of CCM−-PEG. Methoxy-N-(4-nitrophenyloxycarbonyl)amino-PEG was generated from a reaction between methoxyamino-PEG (980 mg, 1.3 mmol) in 10 mL of dry tetrahydrofuran (THF) and a 2 mL solution of bis(4-nitrophenyl)carbonate (BNPC) (37.2 mg) and N,N-diisopropylethylamine (DIEA) (13.3 μL) in dry THF for 15 h, followed by an additional 2.5 h of incubation with a solution of BNPC (10.5 mg) and DIEA (21 μL) at room temperature under an argon perfusion. The purified methoxy-N-(4-nitrophenyloxycarbonyl)amino-PEG (500 mg) dissolved in 4 mL of dry dimethylformamide (DMF) containing 17.4 μL of DIEA was incubated with CCM (36.8 mg) in 2 mL of the same solvent for 3 days at room temperature. The CCM−PEG generated from this reaction was then purified by column chromatography on silica gel. This reaction generated CCM−PEG with 40% yield and a calculated MW of 1145. The identification and characterization of newly synthesized CCM−PEG were performed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS), reversed-phase high-performance liquid chromatography (RP-HPLC), and ^{1}H nuclear magnetic resonance (^{1}H NMR) analyses (*46*).

Cell Culture and Differentiation of 3T3-L1 Preadipocytes. The 3T3-L1 preadipocyte cell line was obtained from American Type Culture Collection. DMEM containing 10% (v/v) FCS was used to maintain 3T3-L1 preadipocytes. To differentiate 3T3-L1 preadipocytes to mature adipocytes, at day 0 when 3T3-L1 preadipocytes were 2 days post confluent, cells were stimulated to differentiate with DMEM supplemented with 10% FBS and adipogenic cocktail (i.e., 167 nM insulin, 0.5 mM IBMX, and 5 μM DEX) for 2 days. Cells were then cultured in 10% FBS−DMEM with insulin for another 2 days, followed by an additional 2 days of culture with 10% FBS−DMEM, at which time > 90% of cells were differentiated into mature adipocytes. All media included 100 U/mL penicillin, 100 μg/mL streptomycin, and sodium pyruvate. Cells were cultured in a humidified 5% CO_2 incubator at 37 °C. To determine the effect of CCM dissolved in DMSO (CCM−DMSO) or CCM−PEG dissolved in phosphate-buffered saline (PBS) on adipogenesis, 2 days postconfluent 3T3-L1 preadipocytes were differentiated in the presence or absence of various concentration (0−30 μM) of CCM−PEG or CCM−DMSO for 6 days. At day 6, cells were subjected to either Oil Red O staining for a quantitative analysis of intracellular lipids in adipocytes or real-time PCR for a quantitative analysis of adipogenic gene expression. DMSO concentration in 3T3-L1 cells

was maintained at 0.001%, which did not show any cytotoxicity as evidenced by MTT assay (data not shown).

Detection of Intracellular CCM. Intracellular CCM was monitored and quantified by fluorescence spectrophotometry and confocal microscopy. Proliferating 3T3-L1 preadipocytes or differentiated 3T3-L1 adipocytes for 6 days were incubated with various concentrations (0−30 μM) of CCM−PEG, CCM−DMSO, or their vehicles (e.g., PBS and DMSO) to examine the effect of CCM−DMSO or CCM−PEG on intracellular levels of CCM. Proliferating 3T3-L1 preadipocytes or differentiated 3T3-L1 adipocytes for 6 days were also incubated with 20 μM CCM−PEG, CCM−DMSO, or their vehicles for the indicated time points to determine the effect of CCM−DMSO or CCM−PEG on cellular retention of CCM. After the incubation, medium was removed and the cells were washed twice with PBS followed by trypsinization and resuspension in PBS. Because both CCM and CCM−PEG exhibit fluorescence, fluorescence microscopy has been previously used to detect CCM (*47*) and CCM−PEG (*48*) in the cells. The fluorescence of CCM in 3T3-L1 cells was measured by fluorescence spectrometer (Molecular Devices Inc., Chicago, IL) using excitation at 414 nm and emission at 538 nm. Cells treated with either PBS or DMSO were used as control for fluorescence measurements. To visualize intracellular CCM, confocal microscopy analysis was conducted using 3T3-L1 cells cultured in a glass-bottomed culture plate in the presence or absence of CCM−DMSO and CCM−PEG. These cells were subjected to confocal microscope, the Zeiss LSM 710 confocal laser scanning microscope (Zeiss LSM 710 confocal laser scanning microscope, Carl Zeiss Optical Inc., Chester, VA). The following settings were used for capturing CCM signal: 488 nm argon laser power 5%, the pinhole at 1 Airy unit (38 μm), 488 nm filter, gain 603, and pixel dwell time of 6.3 s. The objective used was a C-Apochromat ×40/1.20 W correction UV−vis-IR M27. Epifluorescence, brightfield, and DIC images were taken with a Nikon Eclipse 800, mercury arc lamp, ex 488, BA 515-530.

Cell Viability Assay. The MTT assay was employed to determine the effect of CCM−DMSO and CCM−PEG on the viability of 3T3-L1 cells. Proliferating 3T3-L1 preadipocytes or differentiated 3T3-L1 adipocytes treated with different concentrations of CCM in 10% FCS-containing medium for 24 h were further incubated with fresh medium containing MTT solution (0.5 mg/mL) for 1 h at 37 °C. The violet precipitate (formazan) in MTT-treated cells was dissolved in DMSO and quantified by a microplate reader (Beckman Coulter, Brea, CA) at a wavelength of 595 nm.

Oil Red O (ORO) Staining. To monitor the levels of intracellular lipids in differentiated adipocytes in the presence or absence of CCM−DMSO or CCM−PEG for 6 days, ORO staining was performed in differentiated 3T3-L1 adipocytes at day 6 as described elsewhere (*33*). The ORO-stained cells were visualized by light microscopy and photographed. Undifferentiated preadipocytes served as negative control. The ORO-stained lipids extracted by isopropanol from differentiated adipocytes were subjected to spectrophotometric analysis at a wavelength of 490 nm for a quantitative analysis of intracellular lipid accumulation. The half-maximal inhibitory concentration (IC_{50}) was determined by the concentration of CCM that provides 50% inhibition of adipogenesis.

Isolation of Total RNA and Quantitative Reverse Transcription (RT)−Polymerase Chain Reaction (PCR). RNA extraction and cDNA generation were performed using Trizol reagent and a SuperScriptII kit by following the manufacturer's instructions. Newly synthesized cDNA was subjected to quantitative RT-PCR reactions using a StepOne Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The primers used in this study were designed by Primer Express3.0 software (Applied Biosystems) and are shown in **Table 1**. The signals were normalized to β-actin, and the results were expressed as relative fold of induction.

Statistical Analysis. Data are shown as means ± SEM. Statistical analysis was performed using SAS9.2 software. One-way ANOVA was used to determine the significance of treatment effect and interactions. Significant differences between group means were assessed by Bonferroni's method and were accepted at $P < 0.05$.

RESULTS AND DISCUSSION

Dose-Dependent Cellular Uptake of CCM−PEG and CCM−DMSO in 3T3-L1 Cells. The water-soluble CCM−PEG conjugates have been shown to improve cytotoxicity in human cancer cells potentially through their increased cell internalization ability (*46*).

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Table 1. Primer Sequences Used for Quantitative PCR

| gene		5′−3′ sequence
C/EBPβ	F[a]	AGC GGC TGC AGA AGA AGG T
	R[a]	GGC AGC TGC TTG AAC AAG TTC
PPARγ	F	CCC AAT GGT TGC TGA TTA CAA AT
	R	TTT CTA CTT GAT CGC ACT TTG GTA TTC T
C/EBPα	F	GGT TTA GGG ATG TTT GGG TTT TT
	R	AAG CCC ACT TCA TTT CAT TGG T
adiponectin	F	GAT GCA GGT CTC TTG GTC CTA A
	R	GGC CCT TCA GCT CCT GTC A
leptin	F	CAC ACA CGC AGT CGG TAT CC
	R	AGC CCA GGA ATG AAG TCC AA
resistin	F	TGC CAG TGT GCA AGG ATA GAC T
	R	CGC TCA CTT CCC CGA CAT
β-actin	F	AGA TGA CCC AGA TCA TGT TTG AGA
	R	CAC AGC CTG GAT GGC TAC GT

[a] F, forward; R, reverse.
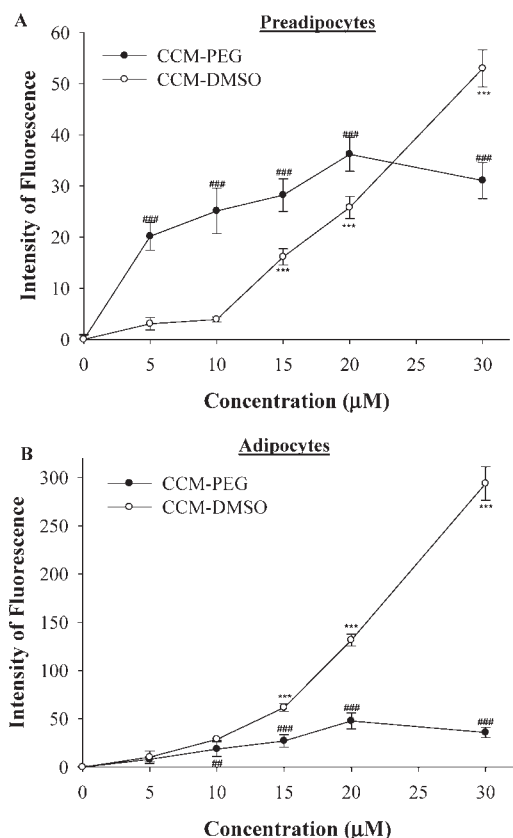
Figure 1. Dose-dependent cellular uptake of CCM−PEG and CCM−DMSO in 3T3-L1 preadipocytes and adipocytes: fluorescence spectrophotometric analysis of intracellular CCM in proliferating 3T3-L1 preadipocytes (**A**) and differentiated 3T3-L1 adipocytes (**B**) after incubation with various concentrations of CCM−PEG, CCM−DMSO, or their vehicles (e.g., PBS and DMSO) for 6 and 2 h, respectively. $n = 12$. ∗∗∗, $P < 0.001$ as compared to cells treated with DMSO. ###, $P < 0.001$ as compared to cells treated with PBS.

These results suggest that CCM−PEG might be suitable for an effective treatment of some human diseases. Although CCM has been recently recognized as a potential antiadipogenic and antiobesity
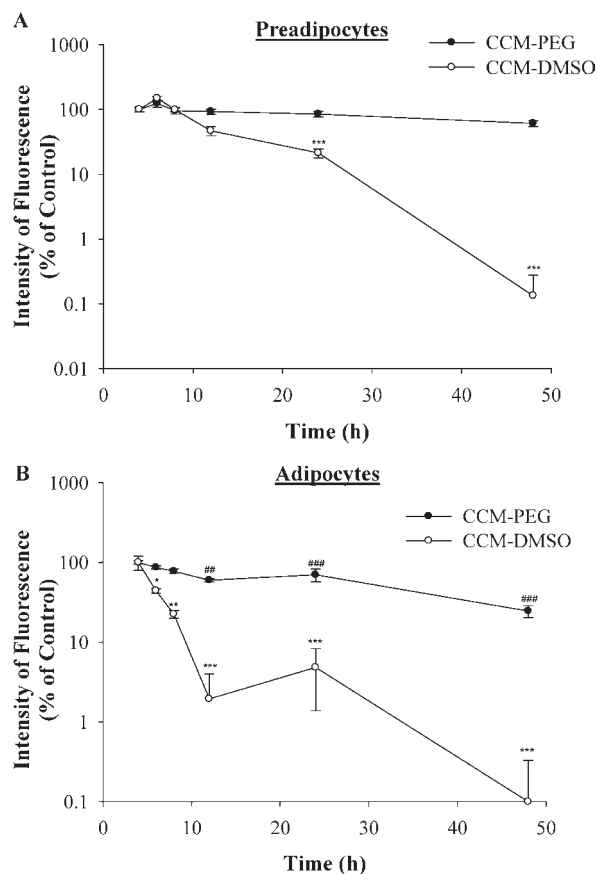

Figure 2. Effect of incubation time on intracellular retention of CCM−PEG and CCM−DMSO in 3T3-L1 preadipocytes and adipocytes: fluorescence spectrophotometric analysis of intracellular CCM in proliferating 3T3-L1 preadipocytes (**A**) and differentiated 3T3-L1 adipocytes (**B**) after incubation with 20 μM CCM−PEG, CCM−DMSO, or their vehicles for the indicated time points. $n = 12$. ∗, $P < 0.05$; ∗∗, $P < 0.01$; and ∗∗∗, $P < 0.001$, versus cells treated with DMSO. ##, $P < 0.01$; and ###, $P < 0.001$, versus cells treated with PBS.

dietary component (*27−31, 49*), the beneficial effects of water-soluble CCM−PEG for an improved regulation of adipogenesis and its cellular uptake kinetics in both preadipocytes and adipocytes have not yet been tested. To address this, we first examined the dose-dependent cellular uptake of CCM and CCM−PEG in 3T3-L1 preadipocytes and adipocytes. Various concentrations (i.e., 0−30 μM) of CCM dissolved in DMSO (CCM−DMSO) or CCM−PEG dissolved in PBS were added to proliferating 3T3-L1 preadipocytes and differentiated adipocytes for 6 and 2 h, respectively. CCM is a naturally fluorescent component, and fluorescence-based detection of CCM in aqueous system (*38*), biological samples (*50, 51*), and mammalian cells (*33, 46*) as well as in adipocytes (*33*) has been previously established. Thus, we monitored the intracellular levels of CCM in 3T3-L1 cells delivered by DMSO and pegylation using fluorescence spectrophotometry. We observed that the intracellular fluorescent signals from preadipocytes (**Figure 1A**) and differentiated adipocytes (**Figure 1B**) were proportional to the concentration of CCM−DMSO. However, saturated levels of fluorescent signal were observed in CCM−PEG-treated preadipocytes and adipocytes with a maximum fluorescent intensity at around 20 μM. These results indicate that pegylated CCM is delivered to 3T3-L1 cells in a manner distinct from that of unmodified CCM, probably due to the difference in water solubility and/or molecular weight between these two compounds.
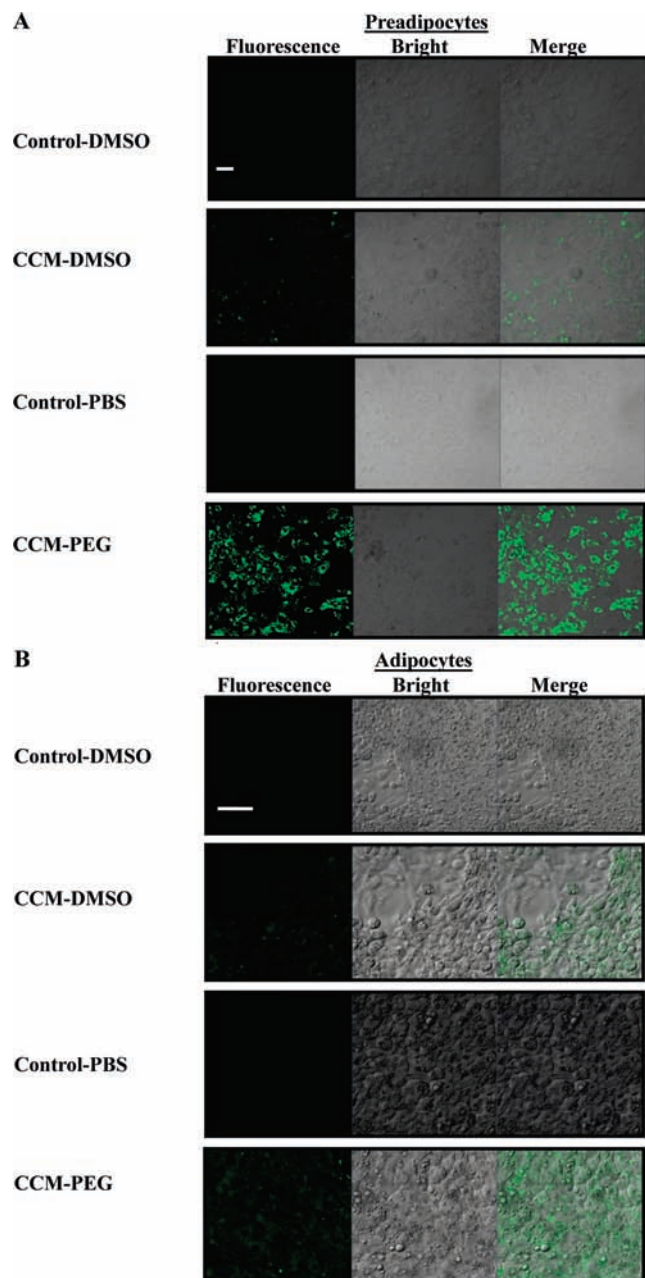
Figure 3. Imaging analysis of intracellular CCM in 3T3-L1 preadipocytes and adipocytes: confocal microscopy of proliferating 3T3-L1 preadipocytes (**A**) and differentiated 3T3-L1 adipocytes (**B**) after incubation with 20 μM CCM−PEG, CCM−DMSO, or their vehicles for 24 h. Scale bar, 50 μm.

Increased Cellular Retention of CCM−PEG in 3T3-L1 Cells. It is possible that the different levels of intracellular fluorescent signal in 3T3-L1 cells treated with CCM−DMSO and CCM−PEG could also result from a rapid degradation and/or change in stability of CCM−PEG in 3T3-L1 cells. To address this, the fluorescence intensity of preadipocytes and adipocytes incubated with 20 μM CCM−DMSO or CCM−PEG at various time points was measured and monitored. The fluorescence signal of intracellular CCM in preadipocytes reached its maximum level within 6 h of incubation, followed by a dramatic decrease to the background level after 48 h of incubation (**Figure 2A**). Similarly, the fluorescence signal of intracellular CCM in adipocytes was rapidly decreased to the background level after 10 h of incubation (**Figure 2B**). Consistent with previous reports of the rapid metabolism and degradation of CCM under various conditions and tissues (*52−55*), our results indicate the likelihood of the presence of an active metabolic
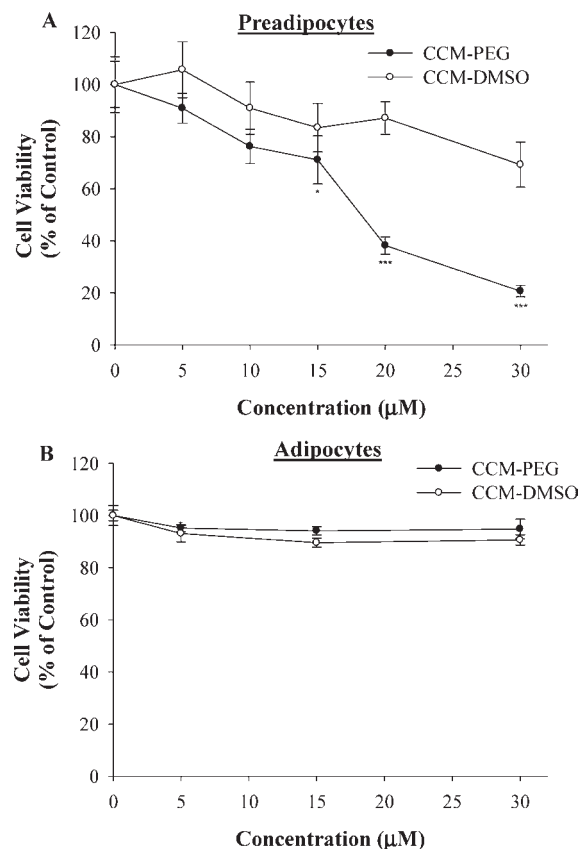

Figure 4. Effect of CCM−PEG and CCM−DMSO on viability of 3T3-L1 cells: cell viability assay of proliferating 3T3-L1 preadipocytes (**A**) and differentiated adipocytes (**B**) after incubation with various concentrations of CCM−PEG or CCM−DMSO for 24 h. $n = 3−9$. $*$, $P < 0.05$; and $***$, $P < 0.001$, as compared to untreated cells.

pathway that mediates a rapid metabolism and/or degradation of the natural form of CCM in both preadipocytes and adipocytes. To the contrary, a consistent level of fluorescence signal was observed from preadipocytes (**Figure 2A**) and adipocytes (**Figure 2B**) incubated with CCM−PEG at different time points. This result suggests that the sustained fluorescence signal of intracellular CCM−PEG in 3T3-L1 cells during the 48 h incubation period is likely due to enhanced resistance of PEG-conjugated CCM against cellular metabolism and/or other intracellular degradation processes. In supporting of our notion, pegylation has been shown to protect bioactive molecules against enzymatic or proteolytic degradation (*56*, *57*). Moreover, pegylated resveratrol, a hydrophobic phytochemical, was shown to enhance absorption and resistance to enzymatic hydrolysis in the intestinal environment (*44*). To further investigate the different cellular retention profiles between intracellular CCM and CCM−PEG in 3T3-L1 cells, preadipocytes and adipocytes treated with either DMSO, PBS, CCM−DMSO, or CCM−PEG at 20 μM for 24 h were subjected to confocal microscopy (**Figure 3**). Consistent with our quantitative analysis of intracellular CCM and CCM−PEG shown in **Figure 2**, no significant intracellular fluorescent emission signal was observed in preadipocytes (**Figure 4A**) and adipocytes (**Figure 4B**) incubated with CCM−DMSO. However, the fluorescent emission signals from preadipocytes and adipocytes incubated with CCM−PEG were higher than those from cells incubated with CCM−DMSO. In particular, an enhanced fluorescence signal of intracellular CCM−PEG was observed in preadipocytes compared with that in adipocytes. This result suggests an effective internalization of CCM−PEG in preadipocytes compared to adipocytes, probably due to improved water solubility. It is possible that our fluorescence-based analysis of intracellular CCM could recognize

not only the intact CCM but also other fluorescent CCM metabolites in 3T3-L1 cells. In support, our high-performance liquid chromatography—mass spectrometry (HPLC-MS) analysis detected both the
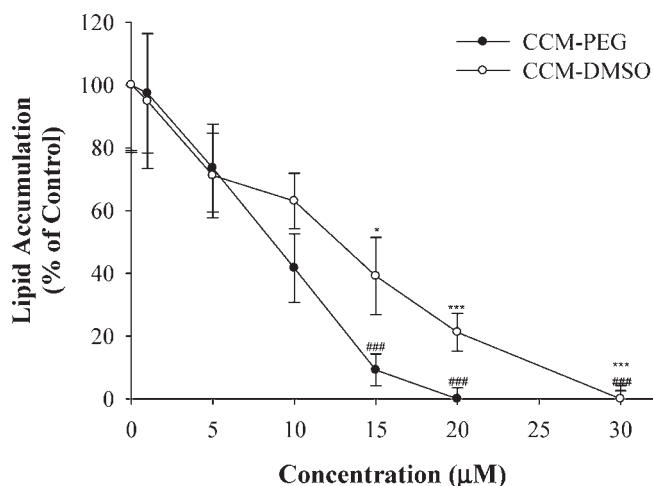

Figure 5. Effect of CCM—PEG and CCM—DMSO on adipogenesis of 3T3-L1 cells: quantitative analysis of intracellular lipid accumulation in 3T3-L1 adipocytes after differentiation with various concentrations of CCM—PEG or CCM—DMSO for 6 days. $n = 6$. *, $P < 0.05$; and ***, $P < 0.001$, versus cells treated with DMSO. ###, $P < 0.001$ versus cells treated with PBS.

intact form of CCM and a number of CCM metabolites in preadipocytes incubated with CCM—DMSO but not in adipocytes. However, the signal of the CCM metabolites in these cells was too weak to quantify compared with that of the intact form of CCM. On the other hand, HPLC-MS detected no signal of free CCM in preadipocytes treated with CCM—PEG (data not shown). This result suggests a potential resistance of intracellular CCM—PEG to hydrolysis in preadipocytes. A more accurate quantitative analysis of time-dependent generation of CCM— and CCM—PEG metabolites in 3T3-L1 cells will broaden our understanding of different cellular uptake kinetics of CCM and CCM—PEG in adipose tissue. Nevertheless, our novel CCM—PEG is likely to be efficiently internalized into 3T3-L1 cells, preferably into preadipocyte, and this is due to, at least in part, enhanced resistance to cellular metabolism and/or degradation through improved water solubility and polymerization of CCM by pegylation.

Effect of CCM—PEG and CCM—DMSO on Viability of 3T3-L1 Cells. CCM exerts numerous biological activities, including an antiproliferative and apoptotic role in cancer cells (20–23, 58). Moreover, our previous study demonstrated an inhibitory function of CCM in the viability of preadipocytes, but differentiating preadipocytes and fully differentiated adipocytes were resistant to CCM-induced cytotoxicity (33). To investigate the functional consequence of enhanced water solubility and cellular internalization of CCM—PEG in 3T3-L1 cells, we first tested the effect of CCM—PEG and CCM—DMSO on the viability of 3T3-L1 cells
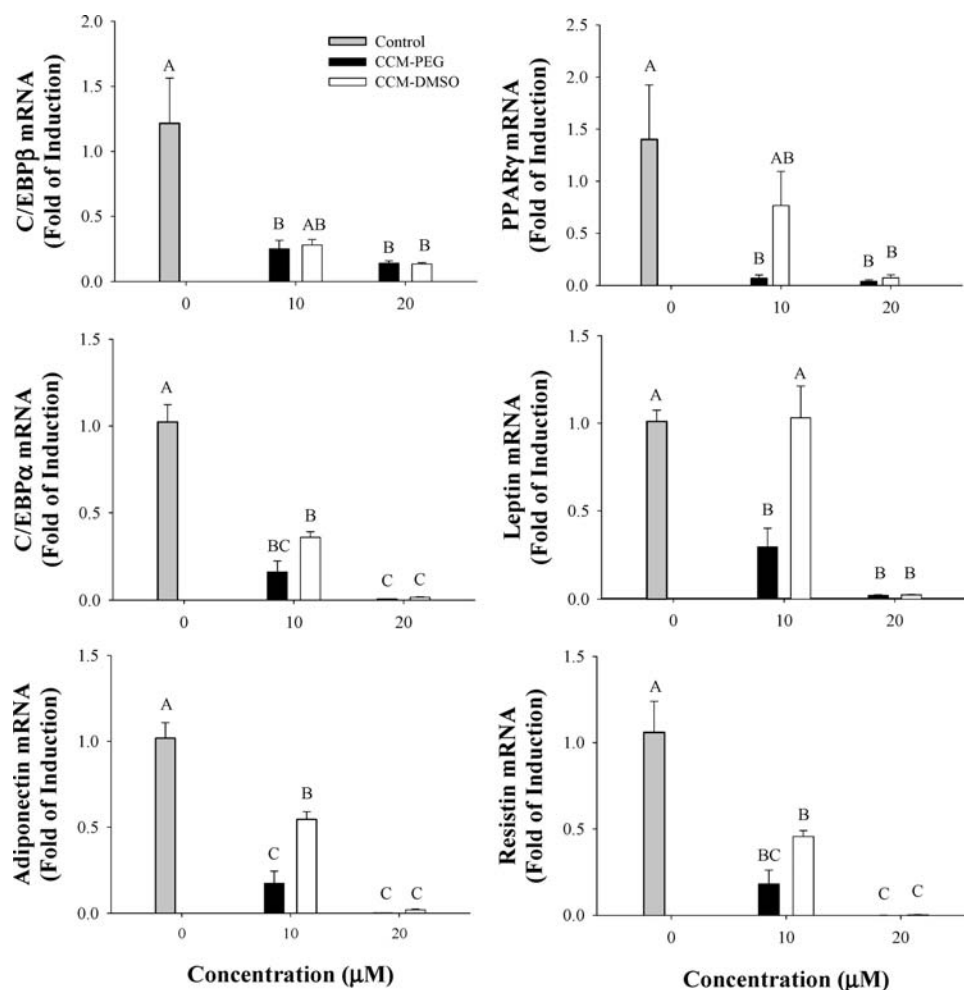

Figure 6. Effect of CCM—PEG and CCM—DMSO on adipogenic gene expression in 3T3-L1 cells: real-time PCR analysis of adipogenic marker genes (C/EBPβ, PPARγ, C/EBPα, leptin, adiponectin, and resistin) in 3T3-L1 adipocytes after differentiation with various concentrations of CCM—PEG or CCM—DMSO for 6 days. $n = 6$. Means that do not share any letters are significantly different at $P < 0.05$.

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at different stages of differentiation by measuring MTT reduction in cells treated with different concentrations of CCM−PEG or CCM−DMSO for 24 h. As shown in **Figure 4A**, CCM−DMSO inhibited the viability of preadipocytes with a 30% decrease in viability at 30 μM CCM. Interestingly, CCM−PEG was shown to be more effective in inhibiting viability of preadipocytes with an 80% decrease in cell viability at 30 μM CCM−PEG. Consistent with our previous study (*33*), CCM−DMSO exhibited less effect on the viability of differentiated adipocytes with a maximum 10% inhibition at 15 μM CCM−DMSO (**Figure 4B**). A similar effect was also shown in differentiated adipocytes treated with CCM−PEG (**Figure 4B**). These results suggest that pegylation of CCM enhances its inhibitory role in the viability of preadipocytes potentially through its improved cytotoxic property.

Effect of CCM−PEG and CCM−DMSO on Adipogenesis of 3T3-L1 Cells. CCM has recently been proposed to be a potential inhibitor in adipogenesis and high-fat diet-induced adiposity in mice (*27*−*31*, *49*). It was, therefore, suggested that CCM functions as a preventive dietary component in the development of obesity. We also demonstrated that treatment of differentiating 3T3-L1 cells with CCM resulted in an inhibition of adipogenesis with no cellular toxic effect (*33*). This prompted us to further test the improved biological activity of CCM−PEG in adipose biology. Thus, we examined the antiadipogenic ability of CCM−PEG and CCM−DMSO in 3T3-L1 preadipocytes. Treatment of differentiating 3T3-L1 preadipocytes with increasing concentrations of CCM−DMSO and CCM−PEG for 6 days resulted in a dose-dependent inhibition of lipid accumulation as quantitatively assessed by ORO staining (**Figure 5**). This analysis revealed the half-inhibitory concentrations (IC$_{50}$) of 7.96 and 12.31 μM for CCM−PEG and CCM−DMSO, respectively (**Figure 5**). Furthermore, almost complete inhibition of adipogenesis was observed in cells treated with 20 μM CCM−PEG and in 30 μM CCM−DMSO. The improved inhibitory effect of CCM−PEG in adipogenesis was also confirmed by quantitative analysis of mRNA levels of adipocyte marker genes, including C/EBPβ, PPARγ, C/EBPα, leptin, adiponectin, and resistin in adipocytes treated with CCM−PEG or CCM−DMSO for 6 days. Treatments of differentiating preadipocytes with 20 μM CCM−PEG and CCM−DMSO exhibited almost complete inhibition of mRNA levels of genes encoding adipogenic transcription factors such as C/EBPβ, PPARγ, and C/EBPα and genes encoding adipokines such as leptin, adiponectin, and resistin (**Figure 6**). However, when cells were differentiated with CCM−PEG and CCM−DMSO at 10 μM, CCM−PEG was more effective in suppressing the mRNA levels of leptin and adiponectin than CCM−DMSO. This result is in agreement with the ORO staining result of an effective inhibitory function of CCM−PEG in lipid-accumulating ability of 3T3-L1 preadipocytes (**Figure 5**). Thus, our results indicate that PEG conjugation of CCM is associated with an improvement of antiadipogenic function of CCM in cultured adipocytes. Although more studies are needed to elucidate the bioavailability of CCM−PEG and its efficacy in regulating adipose development in vivo, evidence suggests that pegylation may also increase resistance of pegylated derivatives to hydrolysis and stability in the gastrointestinal tract, probably due to the steric hindrance of created by PEG polymer (*59*). Moreover, some pegylated compounds with small PEGs have been shown to enhance their transmembrane penetration properties (*60*, *61*). Accordingly, it is plausible that oral administration of water-soluble CCM−PEG as a food ingredient might result in an improvement of its accessibility to the gastrointestinal tract and its circulation to the blood and target tissues in vivo. Taken together, we propose that aqueous solubility and increased cellular retention of CCM−PEG are correlated with its

enhanced antiproliferative property in preadipocytes and antiadipogenic property in differentiating preadipocytes. This will, in turn, contribute to preventing or delaying the development of adipose tissue.

ABBREVIATIONS USED

PEG, polyethylene glycol; CCM, curcumin; C/EBPα, CCAAT/enhancer binding proteinα; PPARγ, peroxisome proliferator-activated receptor γ; DEX, dexamethasone; IBMX, isobutylmethylxanthine; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; BNPC, bis(4-nitrophenyl)carbonate; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; RP-HPLC, reversed-phase high-performance liquid chromatography; NMR, nuclear magnetic resonance; RT-PCR, reverse transcription−polymerase chain reaction.

LITERATURE CITED

(1) Dandona, P.; Aljada, A.; Bandyopadhyay, A. Inflammation: the link between insulin resistance, obesity and diabetes. *Trends Immunol.* **2004**, *25* (1), 4−7.

(2) Kahn, S. E.; Hull, R. L.; Utzschneider, K. M. Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* **2006**, *444* (7121), 840−846.

(3) Bruce, K. D.; Hanson, M. A. The developmental origins, mechanisms, and implications of metabolic syndrome. *J. Nutr.* **2010**, *140* (3), 648−652.

(4) Spiegelman, B. M.; Flier, J. S. Adipogenesis and obesity: rounding out the big picture. *Cell* **1996**, *87* (3), 377−389.

(5) Ntambi, J. M.; Young-Cheul, K. Adipocyte differentiation and gene expression. *J. Nutr.* **2000**, *130* (12), 3122S−3126S.

(6) Sul, H. S.; Smas, C. M.; Wang, D.; Chen, L. Regulation of fat synthesis and adipose differentiation. *Prog. Nucleic Acid Res. Mol. Biol.* **1998**, *60*, 317−345.

(7) Ross, S. E.; Hemati, N.; Longo, K. A.; Bennett, C. N.; Lucas, P. C.; Erickson, R. L.; MacDougald, O. A. Inhibition of adipogenesis by Wnt signaling. *Science* **2000**, *289* (5481), 950−953.

(8) Lee, M. S.; Kim, C. T.; Kim, Y. Green tea (−)-epigallocatechin-3-gallate reduces body weight with regulation of multiple genes expression in adipose tissue of diet-induced obese mice. *Ann. Nutr. Metab.* **2009**, *54* (2), 151−157.

(9) Moon, H. S.; Chung, C. S.; Lee, H. G.; Kim, T. G.; Choi, Y. J.; Cho, C. S. Inhibitory effect of (-)-epigallocatechin-3-gallate on lipid accumulation of 3T3-L1 cells. *Obesity* (*Silver Spring*) **2007**, *15* (11), 2571−2582.

(10) Wolfram, S.; Wang, Y.; Thielecke, F. Anti-obesity effects of green tea: from bedside to bench. *Mol. Nutr. Food Res.* **2006**, *50* (2), 176−187.

(11) Baur, J. A.; Pearson, K. J.; Price, N. L.; Jamieson, H. A.; Lerin, C.; Kalra, A.; Prabhu, V. V.; Allard, J. S.; Lopez-Lluch, G.; Lewis, K.; Pistell, P. J.; Poosala, S.; Becker, K. G.; Boss, O.; Gwinn, D.; Wang, M.; Ramaswamy, S.; Fishbein, K. W.; Spencer, R. G.; Lakatta, E. G.; Le Couteur, D.; Shaw, R. J.; Navas, P.; Puigserver, P.; Ingram, D. K.; de Cabo, R.; Sinclair, D. A. Resveratrol improves health and survival of mice on a high-calorie diet. *Nature* **2006**, *444* (7117), 337−342.

(12) Macarulla, M. T.; Alberdi, G.; Gomez, S.; Tueros, I.; Bald, C.; Rodriguez, V. M.; Martinez, J. A.; Portillo, M. P. Effects of different doses of resveratrol on body fat and serum parameters in rats fed a hypercaloric diet. *J. Physiol. Biochem.* **2009**, *65* (4), 369−376.

(13) Kennedy, A.; Overman, A.; Lapoint, K.; Hopkins, R.; West, T.; Chuang, C. C.; Martinez, K.; Bell, D.; McIntosh, M. Conjugated linoleic acid-mediated inflammation and insulin resistance in human adipocytes are attenuated by resveratrol. *J. Lipid Res.* **2009**, *50* (2), 225−232.

(14) Hsu, C. L.; Yen, G. C. Effects of capsaicin on induction of apoptosis and inhibition of adipogenesis in 3T3-L1 cells. *J. Agric. Food Chem.* **2007**, *55* (5), 1730−1736.

(15) Kang, J. H.; Goto, T.; Han, I. S.; Kawada, T.; Kim, Y. M.; Yu, R. Dietary capsaicin reduces obesity-induced insulin resistance and

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hepatic steatosis in obese mice fed a high-fat diet. *Obesity* (*Silver Spring*) **2010**, *18* (4), 780−787.

(16) Kang, J. H.; Kim, C. S.; Han, I. S.; Kawada, T.; Yu, R. Capsaicin, a spicy component of hot peppers, modulates adipokine gene expression and protein release from obese-mouse adipose tissues and isolated adipocytes, and suppresses the inflammatory responses of adipose tissue macrophages. *FEBS Lett.* **2007**, *581* (23), 4389−4396.

(17) Yang, C. S.; Sang, S.; Lambert, J. D.; Lee, M. J. Bioavailability issues in studying the health effects of plant polyphenolic compounds. *Mol. Nutr. Food Res.* **2008**, *52* (Suppl. 1), S139−S151.

(18) Huang, Q.; Yu, H.; Ru, Q. Bioavailability and delivery of nutraceuticals using nanotechnology. *J. Food Sci.* **2010**, *75* (1), R50−R57.

(19) Aggarwal, B. B.; Sundaram, C.; Malani, N.; Ichikawa, H. Curcumin: the Indian solid gold. *Adv. Exp. Med. Biol.* **2007**, *595*, 1−75.

(20) Ravindran, J.; Prasad, S.; Aggarwal, B. B. Curcumin and cancer cells: how many ways can curry kill tumor cells selectively? *AAPS J.* **2009**, *11* (3), 495−510.

(21) Sharma, R. A.; Euden, S. A.; Platton, S. L.; Cooke, D. N.; Shafayat, A.; Hewitt, H. R.; Marczylo, T. H.; Morgan, B.; Hemingway, D.; Plummer, S. M.; Pirmohamed, M.; Gescher, A. J.; Steward, W. P. Phase I clinical trial of oral curcumin: biomarkers of systemic activity and compliance. *Clin. Cancer Res.* **2004**, *10* (20), 6847−6854.

(22) Steward, W. P.; Gescher, A. J. Curcumin in cancer management: recent results of analogue design and clinical studies and desirable future research. *Mol. Nutr. Food Res.* **2008**, *52* (9), 1005−1009.

(23) Villegas, I.; Sanchez-Fidalgo, S.; Alarcon de la Lastra, C. New mechanisms and therapeutic potential of curcumin for colorectal cancer. *Mol. Nutr. Food Res.* **2008**, *52* (9), 1040−1061.

(24) Jutooru, I.; Chadalapaka, G.; Lei, P.; Safe, S. Inhibition of NFκB and pancreatic cancer cell and tumor growth by curcumin is dependent on specificity protein down-regulation. *J. Biol. Chem.* **2010**, *285* (33), 25332−25344.

(25) Montopoli, M.; Ragazzi, E.; Froldi, G.; Caparrotta, L. Cell-cycle inhibition and apoptosis induced by curcumin and cisplatin or oxaliplatin in human ovarian carcinoma cells. *Cell Prolif.* **2009**, *42* (2), 195−206.

(26) Jagetia, G. C.; Aggarwal, B. B. "Spicing up" of the immune system by curcumin. *J. Clin. Immunol.* **2007**, *27* (1), 19−35.

(27) Weisberg, S. P.; Leibel, R.; Tortoriello, D. V. Dietary curcumin significantly improves obesity-associated inflammation and diabetes in mouse models of diabesity. *Endocrinology* **2008**, *149* (7), 3549−3558.

(28) Asai, A.; Miyazawa, T. Dietary curcuminoids prevent high-fat diet-induced lipid accumulation in rat liver and epididymal adipose tissue. *J. Nutr.* **2001**, *131* (11), 2932−2935.

(29) Seo, K. I.; Choi, M. S.; Jung, U. J.; Kim, H. J.; Yeo, J.; Jeon, S. M.; Lee, M. K. Effect of curcumin supplementation on blood glucose, plasma insulin, and glucose homeostasis related enzyme activities in diabetic db/db mice. *Mol. Nutr. Food Res.* **2008**, *52* (9), 995−1004.

(30) Jang, E. M.; Choi, M. S.; Jung, U. J.; Kim, M. J.; Kim, H. J.; Jeon, S. M.; Shin, S. K.; Seong, C. N.; Lee, M. K. Beneficial effects of curcumin on hyperlipidemia and insulin resistance in high-fat-fed hamsters. *Metabolism* **2008**, *57* (11), 1576−1583.

(31) Ejaz, A.; Wu, D.; Kwan, P.; Meydani, M. Curcumin inhibits adipogenesis in 3T3-L1 adipocytes and angiogenesis and obesity in C57/BL mice. *J. Nutr.* **2009**, *139* (5), 919−925.

(32) Ahn, J.; Lee, H.; Kim, S.; Ha, T. Curcumin-induced suppression of adipogenic differentiation is accompanied by activation of Wnt/β-catenin signaling. *Am. J. Physiol. Cell Physiol.* **2010**, *298* (6), C1510−C1516.

(33) Kim, C. Y.; Le, T. T.; Chen, C.; Cheng, J. X.; Kim, K. H. Curcumin inhibits adipocyte differentiation through modulation of mitotic clonal expansion. *J. Nutr. Biochem.* **2010**, DOI: 10.1016/j.jnutbio. 2010.08.003.

(34) Aggarwal, B. B.; Kumar, A.; Bharti, A. C. Anticancer potential of curcumin: preclinical and clinical studies. *Anticancer Res.* **2003**, *23* (1A), 363−398.

(35) Yen, F. L.; Wu, T. H.; Tzeng, C. W.; Lin, L. T.; Lin, C. C. Curcumin nanoparticles improve the physicochemical properties of curcumin and effectively enhance its antioxidant and antihepatoma activities. *J. Agric. Food Chem.* **2010**, *58* (12), 7376−7382.

(36) Mach, C. M.; Chen, J. H.; Mosley, S. A.; Kurzrock, R.; Smith, J. A. Evaluation of liposomal curcumin cytochrome p450 metabolism. *Anticancer Res.* **2010**, *30* (3), 811−814.

(37) Takahashi, M.; Uechi, S.; Takara, K.; Asikin, Y.; Wada, K. Evaluation of an oral carrier system in rats: bioavailability and antioxidant properties of liposome-encapsulated curcumin. *J. Agric. Food Chem.* **2009**, *57* (19), 9141−9146.

(38) Wang, F.; Wu, X.; Wang, F.; Liu, S.; Jia, Z.; Yang, J. The sensitive fluorimetric method for the determination of curcumin using the enhancement of mixed micelle. *J. Fluorescence* **2006**, *16* (1), 53−59.

(39) Chin, S. F.; Iyer, K. S.; Saunders, M.; St. Pierre, T. G.; Buckley, C.; Paskevicius, M.; Raston, C. L. Encapsulation and sustained release of curcumin using superparamagnetic silica reservoirs. *Chemistry* **2009**, *15* (23), 5661−5665.

(40) Suresh, D.; Srinivasan, K. Tissue distribution and elimination of capsaicin, piperine and curcumin following oral intake in rats. *Indian J. Med. Res.* **2010**, *131*, 682−691.

(41) Dosio, F.; Arpicco, S.; Brusa, P.; Stella, B.; Cattel, L. Poly(ethylene glycol)-human serum albumin-paclitaxel conjugates: preparation, characterization and pharmacokinetics. *J. Controlled Release* **2001**, *76* (1−2), 107−117.

(42) Guerra-Tschuschke, I.; Martin, I.; Gonzalez, M. T. Polyethylene glycol-induced internalization of bacteria into fungal protoplasts: electron microscopic study and optimization of experimental conditions. *Appl. Environ. Microbiol.* **1991**, *57* (5), 1516−1522.

(43) Sugiyama, A.; Sato, A.; Takeuchi, T. PEGylated lactoferrin enhanced its hepatoprotective effects on acute liver injury induced by carbon tetrachloride in rats. *Food Chem. Toxicol.* **2009**, *47* (7), 1453−1458.

(44) Biasutto, L.; Marotta, E.; Mattarei, A.; Beltramello, S.; Caliceti, P.; Salmaso, S.; Bernkop-Schnurch, A.; Garbisa, S.; Zoratti, M.; Paradisi, C. Absorption and metabolism of resveratrol carboxyesters and methanesulfonate by explanted rat intestinal segments. *Cell Physiol. Biochem.* **2009**, *24* (5−6), 557−566.

(45) Liu, X.; Yu, B.; Wang, N.; Zhang, B.; Du, F.; He, C.; Ye, Z. A validated stability-indicating HPLC method for the determination of PEGylated puerarin in aqueous solutions. *J, Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2010**, *878* (23), 2061−2066.

(46) Safavy, A.; Raisch, K. P.; Mantena, S.; Sanford, L. L.; Sham, S. W.; Krishna, N. R.; Bonner, J. A. Design and development of water-soluble curcumin conjugates as potential anticancer agents. *J. Med. Chem.* **2007**, *50* (24), 6284−6288.

(47) Kunwar, A.; Barik, A.; Mishra, B.; Rathinasamy, K.; Pandey, R.; Priyadarsini, K. I. Quantitative cellular uptake, localization and cytotoxicity of curcumin in normal and tumor cells. *Biochim. Biophys. Acta* **2008**, *1780* (4), 673−679.

(48) Anand, P.; Nair, H. B.; Sung, B.; Kunnumakkara, A. B.; Yadav, V. R.; Tekmal, R. R.; Aggarwal, B. B. Design of curcumin-loaded PLGA nanoparticles formulation with enhanced cellular uptake, and increased bioactivity in vitro and superior bioavailability in vivo. *Biochem. Pharmacol.* **2009**, *79* (3), 330−338.

(49) Ryu, M. J.; Cho, M.; Song, J. Y.; Yun, Y. S.; Choi, I. W.; Kim, D. E.; Park, B. S.; Oh, S. Natural derivatives of curcumin attenuate the Wnt/β-catenin pathway through down-regulation of the transcriptional coactivator p300. *Biochem. Biophys. Res. Commun.* **2008**, *377* (4), 1304−1308.

(50) Zhang, J.; Jinnai, S.; Ikeda, R.; Wada, M.; Hayashida, S.; Nakashima, K. A simple HPLC-fluorescence method for quantitation of curcuminoids and its application to turmeric products. *Anal. Sci.* **2009**, *25* (3), 385−388.

(51) Schiborr, C.; Eckert, G. P.; Rimbach, G.; Frank, J. A validated method for the quantification of curcumin in plasma and brain tissue by fast narrow-bore high-performance liquid chromatography with fluorescence detection. *Anal. Bioanal. Chem.* **2010**, *397* (5), 1917−1925.

(52) Wang, Y. J.; Pan, M. H.; Cheng, A. L.; Lin, L. I.; Ho, Y. S.; Hsieh, C. Y.; Lin, J. K. Stability of curcumin in buffer solutions and characterization of its degradation products. *J. Pharm. Biomed. Anal.* **1997**, *15* (12), 1867−1876.

(53) Oetari, S.; Sudibyo, M.; Commandeur, J. N.; Samhoedi, R.; Vermeulen, N. P. Effects of curcumin on cytochrome P450 and glutathione S-transferase activities in rat liver. _Biochem. Pharmacol._ **1996**, _51_ (1), 39−45.

(54) Garcea, G.; Jones, D. J.; Singh, R.; Dennison, A. R.; Farmer, P. B.; Sharma, R. A.; Steward, W. P.; Gescher, A. J.; Berry, D. P. Detection of curcumin and its metabolites in hepatic tissue and portal blood of patients following oral administration. _Br. J. Cancer_ **2004**, _90_ (5), 1011−1015.

(55) Hoehle, S. I.; Pfeiffer, E.; Solyom, A. M.; Metzler, M. Metabolism of curcuminoids in tissue slices and subcellular fractions from rat liver. _J. Agric. Food Chem._ **2006**, _54_ (3), 756−764.

(56) Remaut, K.; Lucas, B.; Raemdonck, K.; Braeckmans, K.; Demeester, J.; De Smedt, S. C. Protection of oligonucleotides against enzymatic degradation by pegylated and nonpegylated branched polyethyleneimine. _Biomacromolecules_ **2007**, _8_ (4), 1333−1340.

(57) Zhang, G.; Han, B.; Lin, X.; Wu, X.; Yan, H. Modification of antimicrobial peptide with low molar mass poly(ethylene glycol). _J. Biochem._ **2008**, _144_ (6), 781−788.

(58) Choudhuri, T.; Pal, S.; Das, T.; Sa, G. Curcumin selectively induces apoptosis in deregulated cyclin D1-expressed cells at G2 phase of cell cycle in a p53-dependent manner. _J. Biol. Chem._ **2005**, _280_ (20), 20059−20068.

(59) Fishburn, C. S. The pharmacology of PEGylation: balancing PD with PK to generate novel therapeutics. _J. Pharm. Sci._ **2008**, _97_ (10), 4167−4183.

(60) Gursahani, H.; Riggs-Sauthier, J.; Pfeiffer, J.; Lechuga-Ballesteros, D.; Fishburn, C. S. Absorption of polyethylene glycol (PEG) polymers: the effect of PEG size on permeability. _J. Pharm. Sci._ **2009**, _98_ (8), 2847−2856.

(61) Asada, H.; Douen, T.; Mizokoshi, Y.; Fujita, T.; Murakami, M.; Yamamoto, A.; Muranishi, S. Stability of acyl derivatives of insulin in the small intestine: relative importance of insulin association characteristics in aqueous solution. _Pharm. Res._ **1994**, _11_ (8), 1115−1120.