RESEARCH ARTICLE

Curcumin Inhibits Expression of Inhibitor of DNA Binding 1 in PC3 Cells and Xenografts

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Abstract

Inhibitor of DNA binding 1 (Id1) plays an important role in genesis and metastatic progression of prostate cancer. We previously reported that down regulation of Id1 by small interfering RNA could inhibit the proliferation of PC3 cells and growth of its xenografted tumors. Curcumin, the active ingredient of turmeric, has shown anti-cancer properties via modulation of a number of different molecular regulators. Here we investigated whether Id1 might be involved in the anti-cancer effects of curcumin *in vivo* and *in vitro*. We firstly confirmed that curcumin inhibited cell viability in a dose-dependent fashion, and induced apoptosis in PC3 cells, associated with significant decrease in the mRNA and protein expression of Id1. Similar effects of curcumin were observed in tumors of the PC3 xenografted mouse model with intraperitoneal injection of curcumin once a day for one month. Tumor growth in mice was obviously suppressed by curcumin during the period of 24 to 30 days. Both mRNA and protein levels of Id1 were significantly down-regulated in xenografted tumors. Our findings point to a novel molecular pathway for curcumin anti-cancer effects. Curcumin may be used as an Id1 inhibitor to modulate Id1 expression.

Keywords: Curcumin - Id1 - prostate cancer - PC3 cells - xenograft - tumor growth

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Introduction

The inhibitor of DNA binding-1 (Id1) belongs to the family of helix-loop-helix (HLH) transcription factor. It controls some cell behavior and tumor aggressiveness as a negative regulator of basic HLH transcription factor (Benezra et al., 1990; Redrado et al., 2013). Upregulation of Id1 is found in several types of human cancers (Rothschild et al., 2011; Redrado et al., 2013). Id1 plays a role as promoter in several important steps during cell malignant progression, including cell proliferation (Cheng et al., 2011; Strong et al., 2013), invasiveness (Ao et al., 2012; Sun et al., 2012), angiogenesis (Mellick et al., 2010) and even associated with prognosis (Sun et al., 2012; Castañon et al., 2013). Id1 has been found to promote prostate cancer progression through various mechanisms. In human prostate cancer, increased expression of Id1 is correlated with Gleason score which represents the degree of differentiation and invasion of cancer cells (Xiaoling et al., 2009; Sharma et al., 2012). Over expression of Id1 is also linked with the shorter survival of patients, reflecting the aggressive nature of Id1 in the progression of prostate cancer (Forootan et al., 2007). In prostate cancer cell lines, Id1 has been proved to promote cell proliferation, migration (Strong N et al., 2013) and angiogenesis (Ling et al., 2005). It also regulates the apoptotic response of cancer cells (Ling et al., 2006). Furthermore, Id1 has been shown to modulate the cell differentiation of bone which is favoring to metastatic bone disease during the progression of prostate cancer (Yuen et al., 2010). Taken together, Id1 plays serious important roles in the carcinogenesis and metastatic progression of prostate cancer. We previously reported that down regulation of Id1 by small interfering RNA could inhibit the proliferation, induce apoptosis of PC3 cells and supress the growth of its exenografted tumors (Yu et al., 2011).

Curcumin (diferuloylmethane) is an orange-yellow component of turmeric (*Curcuma longa*). It has been used not only as a food additive but also as a traditional herbal medicine to treat a variety of inflammatory conditions and chronic diseases throughout Asia since several centuries ago. Over the last half century, curcumin has been revealed of several important functions in extensive researches (Gupta et al., 2013). Some studies have substantiated the potential therapeutic value of curcumin about its anticarcinogenic properties via modulation of a number of different molecular regulators (Zhang et al., 2012). In prostate cancer, several genes have been verified to be changed by curcumin in vivo and in vitro (Cimino et al., 2012). In pre-experiment, we found that curcumin inhibited the mRNA expression of Id1 in PC3 cell lines, reminding that it may be a new pathway for curcumin

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on its anticancer action. In present study, we aimed to investigate whether Id1 was involved in the anti-cancer effects of curcumin in vivo and in vitro.

Materials and Methods

Cell culture and reagents

PC3, an androgen-independent human prostate cancer cell line, obtained from Type Culture Cell Bank of Chinese academy of Sciences (Shanghai, IBCB), were maintained in RPMI 1640 medium (Gebico, MO) supplemented with 10% (v/v) FCS, penicillin (100 units/ml) and streptomycin (100 g/ml) at 37°C, 5% CO_2.

Cell viability assay

Cell viability was measured by the Cell Counting Kit-8 (CCK-8) (Beyotime, China). Briefly, 2000 cells were seeded in 96-well plates and cultured. Cell viability was examined as the kit instruction at 48h after treated with different concentration of curcumin. The optical density was measured at a wavelength of 450 nm on a Labsystem multiskan microplate reader (Merck Eurolab, Dietikon, Schweiz, Switzerland). Experiments were performed in six-fold replicates and repeated at least twice.

Cell apoptosis assays

To evaluate the extent of apoptotic and necrotic cells, flow cytometry was done with Annexin V-FITC Apoptosis Detection Kit (Beyotime, China). PC3 cells were plated at a density of 5×10^6 per 10cm dish for 24 hours. Curcumin (20μm) was added to the cells. At 48hr after treatment, the cells were collected and counted. A total of 10^5 cells were stained with Annexin V-FITC and propidium iodide (PI) as the instruction manual. Flow cytometric analysis was performed by PAS (Particle Analyzing System, Partec, Münster, Germany) equipped with an argon ion laser tuned at 488 nm wavelength. Green FITC-annexin V fluorescence was measured at 530±15 nm, and red PI fluorescence was measured at 600 nm.

In-vivo tumor xenograft study

Male nude mice (BALB/c nu/nu), 4 weeks old, weighing about 15~18g, were purchased from Shanghai Laboratory Animal Center, Chinese Academy Sciences (SLAC, China) and were bred in homoeothermic (25~27°C), constantly humidified (40~60%), dust-free fresh air in a sterilized, specific pathogen-free environment with sterilized standard mouse chow and water ad libitum. The mice were allowed to acclimate to their new surroundings for one week before experimental manipulation. For xenografts, PC3 cells (2×10^7) were suspended in 0.1ml serum free medium mixed with matrigel (1:1) and subcutaneously injected into the right flank of each mouse. When tumors became palpable, their sizes were measured in two dimensions (a and b) using digital Vernier calipers. Tumor volume was calculated using the formula: 1/2 ab^2. When the tumor volume was about 3 mm^3, the mice were randomly divided into two groups (n=6) for curcumin or medium control. Curcumin (100 mg/kg in 0.1 ml medium) was introperitoneal injected once a day for one month. The tumor size was detected by digital Vernier calipers every 6 days. After 24 h from the final injection, all mice were dislocated put to death. The tumors were removed and prepared for RNA and protein isolation, and for embedded in paraffin wax.

All studies were carried out with approved institutional and experimental animal care and usage protocols. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Quantitative Real-time RT-PCR assay

Total RNA was isolated from PC3 cells or tumor tissues using TRIzol reagent (Invitrogen, USA). The reverse transcription reaction was performed using the BcaBESTTM RNA PCR Kit (TaKaRa, Japan). Real-time quantitative PCR was performed using SYBR Premix Ex Tag (perfect Real Time, TaKaRa bio, Japan) on an Applied Biosystems 7500 fast Sequence Detection System. Primer specificity for Id1: 5-acgacatgaacggctgttactcac-3; 5-ctccaactgaaggtccctgatgtag-3. Beta-actin: 5-attgccgacaggatggaga-3; 5-gagtacttgcgctcaggagga-3. Beta-actin was used as a housekeeping gene to normalize gene expression in each sample. Experiments were performed in triplicate for each data point. The mRNA expression levels were calculated by relative quantity using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The data are presented as the n-fold expression difference of each sample.

Western blotting assay

Cell pellets or tumor tissues were lysised in SDS lysis buffer (Beyotime, China) with 1μg/ml leupeptin, 1 μg/ml aprotinin, and 1mM PMSF. Protein suspension from the whole-cell or tumor lysate (15~30 μg) was loaded onto a sodium dodecylsulfate-polyacrylamide gel (SDS-PAGE) for electrophoresis and then transferred to a PVDF membrane (Millipore, China). The membrane was then incubated with primary antibodies against Id1 (1:1000 dilution, Santa Cruz Biotechnology, CA) for 1hr. After washing with TBS-T, the membrane was incubated with secondary antibody against rabbit IgG and the signals were visualized using ECL plus Western blotting system (Pierce, USA) for detection and developed by X-ray film. Relative density of Id1 protein expression was estimated by densitometry.

Immunostaining assay

For immunostaining, four-micrometer serial sections from paraffin embedded tissues were cut, processed, and immunostained with Id1 antibody (1:250 dilution; SantaCruz Biotechnology), followed by appropriate biotinylated secondary antibodies, and finally with con-jugated horseradish peroxidase streptavidin. The sections were then incubated with DAB working solution for 10min at room temperature. Finally, the nucleuses were counterstained with diluted Harris hematoxylin. For quantitative analysis of Id1 protein expression, the immunostaining images were analyzed by Jieda Image software (Jieda, China). The mean staining intensity and the proportion of positive staining cells were automatically detected and added together as relative index.

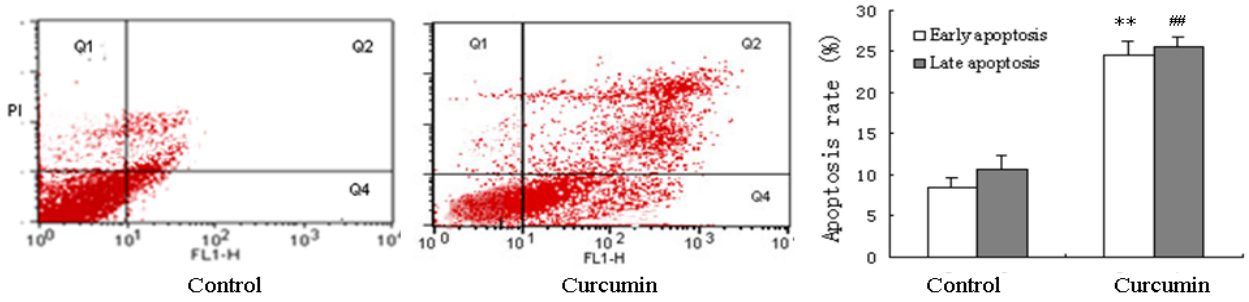

Figure 1. Curcumin Induced the Apoptosis of PC3 Cells. The early or late apoptosis rate was assayed by flow cytometry assessment. PC3 cells were treated by curcumin(20 uM) for 48h and then were collected and double stained by Annexin V and PI. FL1-H: Annexin V-FITC fluorescence; Q1: damaged cells (Annexin V-/PI+); Q2: late apoptotic cells (Annexin V+/PI+); Q3: normal cells (Annexin V-/PI-); Q4: early apoptotic cells (Annexin V+/PI-). All data are expressed as average ± SD. $^{**}P$ <0.01, early apoptosis rate compared with Control; $^{\#\#}P$ <0.01, late apoptosis rate compared with Control

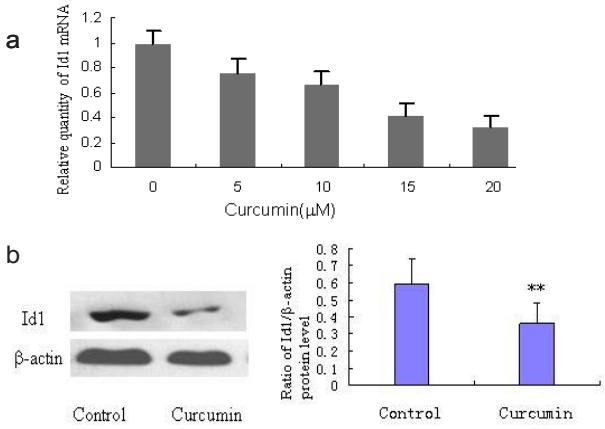

Figure 2. Curcumin Inhibited the Expression of Id1 in PC3 Cells. a. The relative quantity of Id1mRNA was detected by real time RT-PCR assay in PC3 cells treated with different concentration of curcumin for 48h. All data were normalized to β-actin before comparison for fold changes in mRNA levels. Each date was shown with error bar indicating the standard deviation from triplicate experiments. b. The protein of Id1 was detected by Western blotting in PC3 cells after treated by curcumin(20 uM) for 48h. The quantitative data represented as average ± SD was pooled from three separate Western blottings. The intensities for each band were normalized to the constitutively expressed protein of β-actin. $^{**}P$<0.01, compared with control

Statistical analysis

The data were showed as mean ± standard deviation (SD). The difference of measurement data among the groups were analyzed by homogeneity test of variances. T test or Q test was used under an equal condition. A value of P<0.05 was considered to be statistically significant.

Results

Curcumin inhibited the viability of PC3 cells

After treated with curcumin for 48h, the cell viability was detected by CCK-8 assay. The viability of PC3 cells was reduced obviously by curcumin in a dose-dependent fashion. According this, 20μM of curcumin was used in follow-up studies.

Curcumin increased the apoptosis rate of PC3

The early and late apoptosis rate was determined by measuring AnnexinV-FITC and PI double staining after

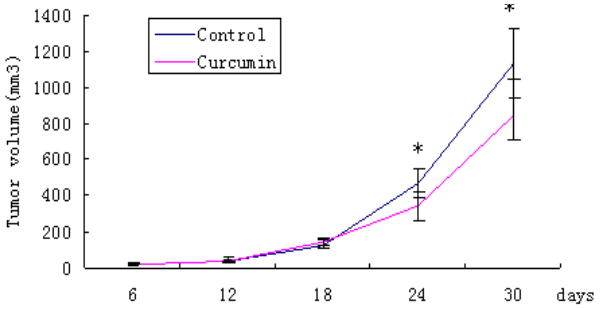

Figure 3. Tumor Growth Curve. When the tumor volume was about 3 mm^3, intraperitoneal injection of curcumin was started every 1 day in 1 month (n=6). Long diameter (a) and short diameter (b) of tumor was measured every 6 days. The volumes (V) of the tumors were calculated according to the formula V = 1/2 ab^2 (mean±SD). $^{*}P$<0.05, compared with Control

curcumin treated in PC3 cells. Results were normalized to values of untreated cells. Representative analysis and regional percentage of an annexin V versus PI dot plot are presented in Figure 1. Early apoptosis (AnnexinV+/PI-) as well as late apoptosis rate (AnnexinV+/PI+) was enhanced obviously after treatment with curcumin (p<0.01).

Curcumin inhibited the expression of Id1 in PC3 cells

The expression of Id1 mRNA and protein in PC3 cells was determined by real time RT-PCR and western blotting assay individually after treated with curcumin for 48h. As shown in Figure 3, the quantity of Id1 mRNA was significantly reduced by curcumin in a dose dependent fashion (Figure 2a). The relative quantity of Id1 protein in curcumin treated group was obviously decreased compared with that in control group (P<0.01) (Figure 2b).

Curcumin suppressed the growth of PC3 xenografts in mice

After curcumin introperitoneal injection, the tumor sizes were measured and calculated every 6 days for one month. As shown in Figure 3, the volume of tumor in curcumin group was obviously smaller than that in control group during curcumin injected for 24 and 30 days (P<0.05).

Curcumin inhibited the expression of Id1 in PC3 xenografted tumors

To explore the effects of curcumin on the expression of Id1 in vivo, the mRNA and protien were isolated

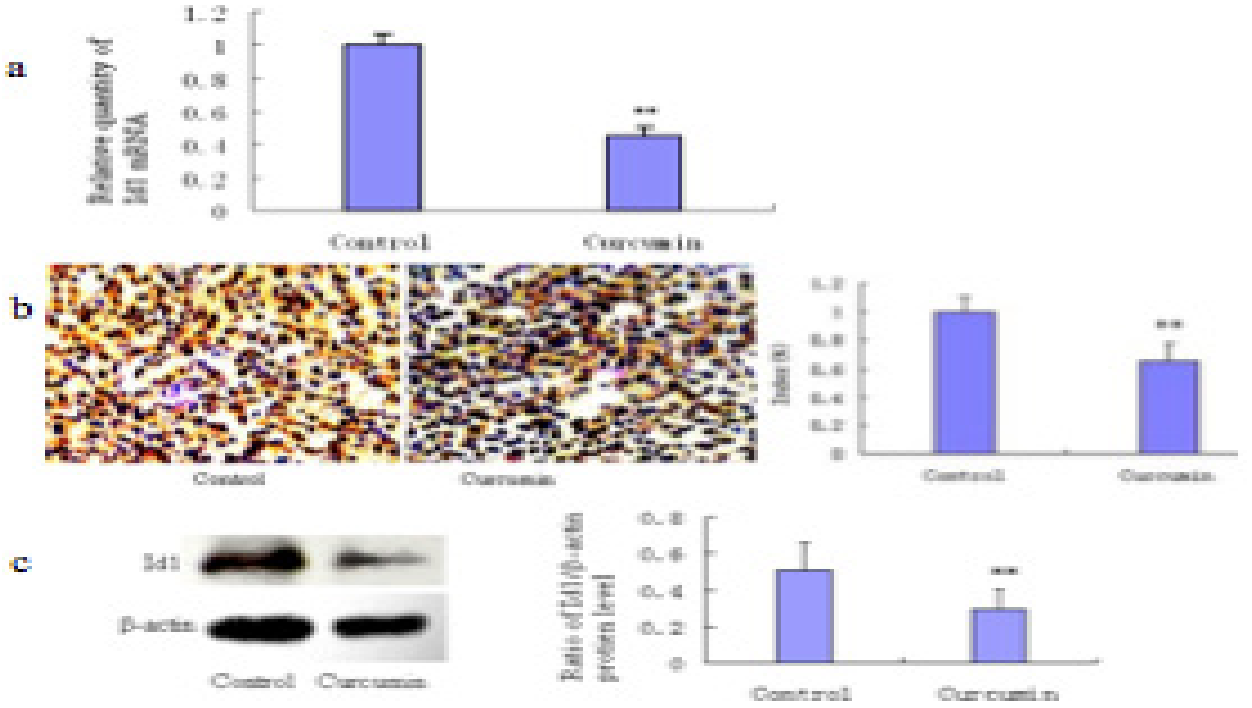

Figure 4. Curcumin Inhibited the Expression of Id1 in PC3 Xenografted Tumors. The mRNA and protien were isolated individually from the tumors in PC3 xenografts after treated by curcumin for 30 days. a. The relative quantity of Id1 mRNA was evaluated by real-time RT-PCR. All data were normalized to β-actin before comparison for fold changes in mRNA levels. The date with error bars indicated the standard deviations from triplicate experiments. ***P*<0.01, compared with control group. b. The expression of Id1 protein was detected by immunohistochemical staining. The representative pictures were taken at magnification 200×. Positive staining for Id1 was mainly in cytoplasm of tumor cells. The index represents the relative quantity of positive Id1 protein, which is the relative sum of positive proportion of cells and the positive density of each protein. All data are expressed as average ± SD from multiplicated experiments. ***P*<0.01, compared with Control. c. The relative quantity of Id1 protein in tumors from western blotting assay. The data are expressed as average ± SD from multiplicated experiments. ***P*<0.01, compared with Control

individually from the tumors in PC3 xenografts after treated by curcumin for 30 days. The relative quantity of Id1 mRNA was evaluated by real-time RT-PCR. The expression of Id1 protein was assayed by immunohistochemistry and Western blotting. More than 50% of Id1 mRNA reduction was found in curcumin treated groups compared with that in controls (P<0.01) (Figure 4a). Similar effects were found in the expression of Id1 protein. As shown in Figure 5b, the positive staining of Id1 protein was of high intensity mainly in the cytoplasm of tumor cells. The immunoassaying index of Id1 protein in the tumors was significantly decreased in curcumin treated group than that in control (P<0.01). The relative quantity of Id1 protein in tumors from western blotting assay was obviously decreased in curcumin treated group compared with that in control group (P<0.01) (Figure 4c).

Discussion

Prostate cancer is the most common solid neoplasm and it is now recognized as one of the most important medical problems facing the male population. Due to its long latency and its identifiable preneoplastic lesions, prostate cancer is an ideal target tumor for chemoprevention. Certainly polyphenolic substance, such as curcumin, represents effective chemoprevention against prostate cancer.

In this study, we confirmed the effects of curcumin on the inhibition of cell viability and the induction of cell apoptosis. We also found that curcumin could inhibit the expression of Id1 mRNA and protien in PC3 cells. The same effect of curcumin on Id1 was further found in its exenografted tumors after treated by curcumin. As we have mentioned above, Id1 plays a key role in prostate cancer carcinogenesis and metastatic progression. In previous study, we found that down regulation of Id1 by small interfere RNA in PC3 cells can decrease cell viability, induce cell apoptosis and senescence (Yu et al., 2011). The similar effects of targeting Id1 mRNA was also found in other kinds of cancer cells (Dong et al., 2011; Soroceanu et al., 2013). So inactivation of Id1 may become a potential intervention in some human cancers. Our results suggest that curcumin may be used as Id1 inhibitors to modulate Id1 expression.

Id1, one member of the family of Ids (Id1-4), has highly conserved dimerization motif known as the HLH domain that acts as a negative regulator of basic HLH (bHLH) transcription factors. In signaling pathways, Id1 plays an important role in cellular development, proliferation and differentiation. The mechanism of Id1 protein is to antagonize bHLH proteins, thereby preventing them from binding to DNA and inhibiting transcription of cellular differentiation-associated genes in cancer. Up to now, it is not clear about the signaling pathway of Id1 in prostate cancer cells. There have some researchs on interfering several genes can modulate Id1 and inhibit the proliferation or induce apoptosis in prostate cancer cells. Bcl-3 knockdown reduced the abundance

of Id1 and Id2 proteins and boosted PCa cells to be more receptive to undergoing apoptosis following treatment with anticancer drug (Ahlqvist et al., 2013). E2A (TCF3) is a multifunctional bHLH transcription factor. Silencing of E2A in prostate cancer DU145 and PC3 cells led to a significant reduction in proliferation due to G1 arrest that was in part mediated by decreased Id1 (Patel and Chaudhary, 2012). Recently, peptide 3C, an inhibitor of Id1 was reported shown good affinity to Id1 protein and exhibited inhibitory effects in cancer cells (Hsiao et al., 2013).

Although the above experiments have good effects on inhibiting of Id1 and cancer cells, the application prospect for clinical use is greatly limited due to their complex experimental process. As our known, for clinical usage of targeting some tumor relative genes, simple and practical compound from natural plant with low or no toxicity has more superior to experimental molecular methods. To date, no studies have reported any toxicity associated with the use of curcumin in either animals or humans (Goel and Aggarwal, 2010). The lack of side effects of curcumin showed a potential strategy in cancer chemoprevention.

The chemopreventive properties of curcumin in prostate cancer cells are attributed to its effects on several targets including transcription factors, growth regulators, apoptotic genes, angiogenesis regulators, and cellular signaling molecules (Ni et al., 2012; Cheng et al., 2013). Curcumin has the ability to inhibit cell growth and induce apoptosis in both androgen-dependent and androgen-independent prostate cancer cell lines acting through the regulation of apoptosis relative signaling (Hilchie et al., 2010; Wei et al., 2013) and other crucial proteins (Teiten et al., 2012; Horie, 2012; Kang et al., 2013). By targeting the inflammatory cytokines CXCL-1 and -2, curcumin also inhibited prostate cancer metastasis in vivo (Killian et al., 2012).). There is no report about curcumin in modulation of Id1 signaling in prostate cancer or other cancer cells. Our results not only found curcumin inhibit the expression of Id1 mRNA and protien in vitro, but also confirmed the same effect of curcumin on Id1 in vivo.

It is known that Id1 is regulated by the TGF-β super-family (Kang et al., 2003; Liang et al., 2009). Smad3 and ATF binding elements in the Id1 promoter mediate this regulation. It has been reported that curcumin inhibits TGF-β-induced Smad2/3 phosphorylation and transcription in human lung cancer H358 and A549 cells (Datta et al., 2013). Curcumin also inhibits the TGF-β-stimulated PTHrP secretion in human breast cancer cells independent of effects on cell growth inhibition, which reveal the reduction in phospho-Smad2/3 and Ets-1 protein levels (Wright et al., 2013). We guess that the inhibition of cucumin on Id1 in our study may also be connection with the changes of TGF-β signaling pathway. More details need to be explored in further research.

Recently, curcumin-loaded nanoparticles (Cum-NPs) prepared with amphilic methoxy poly (ethylene glycol)-polycaprolactone (mPEG-PCL) block copolymers was reported to change the poor solubility of curcumin and to produce superior antitumor effcacy in the treatment of lung cancer in vivo and in vitro (Yin, et al.2013) providing a most promising way in using of curcumin for anticancer.

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The author (s) declare that they have no competing interests.

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