Apoptosis-inducing effects of curcumin derivatives in human bladder cancer cells

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Our aim was to prepare curcumin derivatives and study their apoptosis-inducing effects on bladder cancer cells in order to establish a basis for targeted chemotherapy of cancer. *n*-Maleoyl-L-valine-curcumin (NVC) and *n*-maleoyl-glycine-curcumin (NGC) were chemically synthesized. Intracellular esterase activity of the human bladder cancer EJ cell line and renal tubular epithelial (HKC) cells was examined by 6-carboxyfluorescein diacetate fluorometry. After incubation with NVC or NGC for 6–24 h, cell viability was detected by MTT colorimetry. Cell apoptosis and apoptotic rates were measured by acridine orange/ethidium bromide staining, TUNEL labeling and flow cytometry. Intracellular caspase-3 activities were determined by spectrophotometry. The esterase activity within EJ cells was 10.2-fold higher than that of HKC cells, which was abolished by bis-*p*-nitrophenylphosphate, an esterase inhibitor, resulting in decreases in NVC- and NGC-mediated cell viability arrest. For EJ cells, the IC_{50} values of NVC (20.1 μmol/l) and NGC (18.7 μmol/l) were close to curcumin (16.5 μmol/l). Meanwhile, their IC_{50} values on HKC cells were, respectively, 4.06- and 3.23-fold higher than curcumin. Moreover, NVC and NGC induced apoptosis of EJ cells by 10.13–23.36 and 12.42–28.56%, respectively. Administration of these two derivatives resulted in decreased apoptosis of HKC cells compared with curcumin. The caspase-3 activities of EJ cells, but not of HKC cells, were 5.21- and 5.63-fold enhanced by NVC and NGC, respectively. Thus, novel esterase-sensitive curcumin derivatives were synthesized, which induced extensive apoptosis of bladder cancer EJ cells, but not normal cells. *Anti-Cancer Drugs* 17:279–287 © 2006 Lippincott Williams & Wilkins.

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Introduction

In recent years, drugs of plant origin have received much attention due to their enormous potential in the prevention and treatment of cancer [1]. Curcumin, also named diferuloylmethane, is a β-diketone constituent of turmeric, which is obtained from the powdered root of *Curcuma longa* L. [2]. It is used as a spice to give specific flavors and yellow colors to curry, which is consumed daily by millions of people [2]. Curcumin has been used as a traditional medicine for liver disease (jaundice), indigestion, urinary tract diseases, rheumatoid arthritis and insect bites [3–5]. Among the different pharmacological activities of curcumin, such as its anti-inflammatory and anti-oxidative properties, its anti-carcinogenic properties seem to raise the most interest [6]. These properties have been demonstrated by its inhibition of tumor initiation induced by benz[*a*]pyrene or 7,12-dimethylbenz[*a*]anthracene [7] and tumor promotion by phorbol esters in animals [8,9]. Administration of curcumin significantly inhibits carcinogenesis of the forestomach, duodenum, colon and tongue in mice and rats [10,11]. Therefore, curcumin has been entered into phase I clinical trials for chemoprevention by the National Cancer Institute [12].

One potential problem with the clinical use of curcumin is its low potency and poor absorption characteristics [13]. In the past few years, studies of curcumin derivatives have been actively pursued. The naturally occurring derivatives demethoxycurcumin and bis-demethoxycurcumin have shown anti-oxidant and free radical scavenging properties [14,15]. They are effective inhibitors of phase I metabolizing enzymes like cytochrome P450 1A1, 1A2 and 2B1 [16]. Demethoxycurcumin and tetrahydrocurcumin are also known as potent inhibitors of angiogenesis [17,18]. From cDNA microarray studies, the anti-angiogenic activity of demethoxycurcumin was found to inhibit the expression of matrix metalloprotease-9, which is a major mediator of angiogenesis [17]. Hydrazinocurcumin, a novel synthetic curcumin derivative, is a potent inhibitor

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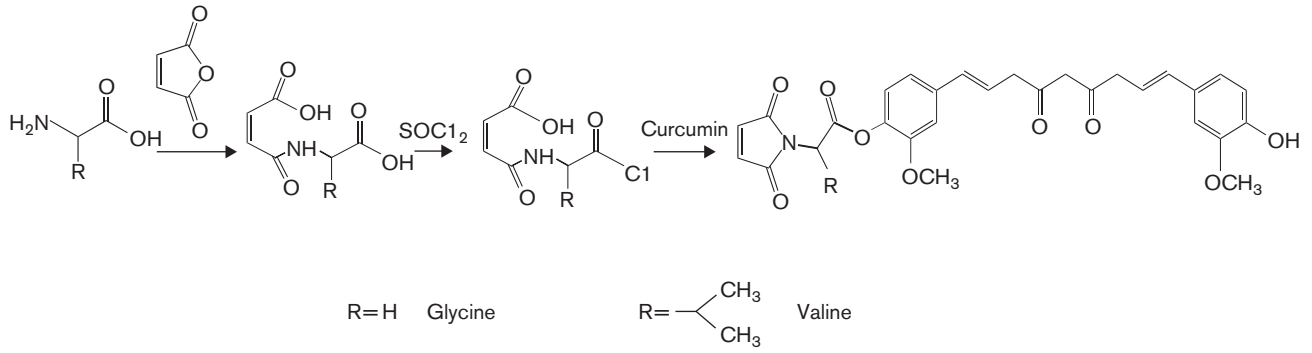
Fig. 1



Preparation of curcumin derivatives, and their identification by IR and NMR. Based on the molecular structure of curcumin, the *n*-maleoyl-L-valine and *n*-maleoyl-glycine groups were covalently linked with curcumin via ester bonds, resulting in the curcumin derivatives NVC and NGC, which were validated by IR spectroscopy, ^{1}H-NMR and ^{13}C-NMR. The molecular formulas of these compounds were $C_{31}O_9H_{31}N$ and $C_{28}O_9H_{25}N$, respectively, which were consistent with design and synthesis methods.

of endothelial cell proliferation [19]. To improve the bioavailability, Kumar *et al.* synthesized several smaller derivatives of curcumin that lack the dieneone structure. These derivatives are more soluble than curcumin, while potentially retaining curcumin's favorable biologic activities, including inhibition of growth of prostate cancer cells [20].

Although it has been indicated that curcumin and its derivatives are not toxic even at high doses in laboratory animals [21], some reports demonstrated their cytotoxicity in normal cells [22–24]. Curcumin was proven to stimulate oxidative damage caused by quercetin in rat hepatocytes [22]. Using single-cell gel electrophoresis (the Comet assay), a highly sensitive technique to analyze DNA damage, Blasiak *et al.* reported that curcumin does not inhibit the DNA-damaging action of hexavalent chromium, a well-recognized carcinogen, in human lymphocytes and gastric mucosa cells [23,24]. Curcumin itself induces DNA damage in these cells [23,24]. Therefore, it is necessary for us to improve the selectivity of curcumin-meditated anti-cancer properties. In the present study, based on the difference in intracellular esterase activity between tumor cells and normal cells, we prepared esterase-sensitive curcumin derivatives, and investigated their effects on growth and apoptosis of human bladder cancer cells and renal tubular epithelial cells, in the hope of developing novel derivatives of curcumin.

Materials and methods

Reagents

Curcumin, MTT, 6-carboxyfluorescein diacetate (6-CFDA), bis-*p*-nitrophenylphosphate (BNPP), ethidium bromide (EB), acridine orange (AO) and propidium iodide were obtained from Sigma (St Louis, Missouri, USA); RPMI 1640 medium and FBS were purchased from Gibco (Grand Island, New York, USA). The *in situ* terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) and caspase-3 activity assay kits were obtained from BD Biosciences (San Jose, California, USA) and Promega (Madison, Wisconsin, USA), respectively.

Preparation of curcumin derivatives

As shown in Fig. 1, based on the molecular structure of curcumin, the *n*-maleoyl-glycine and *n*-maleoyl-L-valine groups were covalently linked with curcumin to form ester bonds. The curcumin derivatives, named *n*-maleoyl-L-valine-curcumin (NVC) and *n*-maleoyl-glycine-curcumin (NGC), were chemically synthesized, and validated by IR and NMR spectroscopy.

Cell culture

The human bladder cancer EJ cell line and renal proximal tubular epithelial cells (HKC) were obtained from the Institute of Urology, Peking University (Beijing, China). Cells were grown in RPMI 1640 medium containing 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 5% CO_2.

Intracellular esterase activity assay

6-CFDA was prepared as stock solutions at a concentration of 10 mmol/l in anhydrous DMSO and stored at −20°C. The determination of intracellular esterase activity was performed according to a previous report [25]. Briefly, confluent monolayers of EJ and HKC were trypsinized, and 3×10^3 viable cells suspended in 100 μl culture medium supplemented with 10% FBS were added to each well of 96-well plates. After the cell density reached 80–90%, the cells were pre-treated with BNPP [26], washed twice with Dulbecco's PBS and incubated with 2 μmol/l 6-CFDA for 30 min at 37°C. The fluorescence, indicating intracellular esterase activity,

was measured at an excitation wavelength of 492 nm and an emission wavelength of 518 nm using a Shimadzu RF5000U fluorometer (Mandel Scientific, Guelph, Ontario, Canada).

Cell viability

Cell viability was monitored by MTT colorimetry. EJ and HKC cells at logarithmic growth phases were seeded into triplicate wells of 96-well plates at a density of 4×10^4 viable cells/ml. Untreated blank control, NVC (10, 20 and 40 μmol/l), NGC (10, 20 and 40 μmol/l) and curcumin (10, 20 and 40 μmol/l) treatment groups were designed for this experiment. After treatment for 6, 12, 18 and 24 h, 20 μl of MTT (5 mg/ml) was added to each well. After 4 h of incubation at 37°C, the cell supernatants were discarded, MTT crystals were dissolved with DMSO and the absorbance measured at 570 nm ($A_{570\,nm}$). Cellular viability inhibition rates were calculated as (%) = (1 – average $A_{570\,nm}$ value of experimental group/ average $A_{570\,nm}$ value of untreated control group) × 100%. The 50% inhibitory concentrations (IC_{50}s) of a 24-h exposure, defined as the drug concentration resulting in 50% reduction of cell viability compared to the untreated control, were then determined by Bliss software.

Cellular morphological observation

Morphological evidence of apoptosis was obtained using AO/EB staining [27]. Briefly, after treatment with 20 μmol/l NVC or NGC for 12 h, cells were harvested with 0.125% trypsin and 0.01% EDTA, resuspended in 95 μl of RPMI 1640 medium, and incubated with 5 μl of AO/EB staining solution (100 mg/l PBS of each dye) at room temperature for 15 min. Cells were examined using fluorescence microscopy and photographed (Olympus, Tokyo, Japan). Viable cells were colored green with intact nuclei. Non-viable cells had bright orange chromatin. Apoptosis was demonstrated by the appearance of cell shrinkage with condensation and fragmentation of the nuclei. Necrotic cells appeared orange with a normal nuclear structure. The numbers of normal (VN), apoptotic but with intact cell membrane (VA), incompetent cell membrane and apoptotic (NVA), and incompetent cell membrane but not apoptotic (NVN) cells were determined by counting five randomly selected high-power (× 200) fields. Apoptosis rates were calculated as (%) = (VA + NVA)/(VN + NVN + VA + NVA) × 100%.

TUNEL assay

Cell slides were prepared from each group. The TUNEL assay was performed according to the manufacturer's protocol. Briefly, after fixation, blockage and transparency, 50 μl FITC-labeling DNA fragmentation reaction solution was added to the slides and incubated at room temperature for 60 min. Then, the slides were incubated with transfer solution at 37°C for 30 min. Under a fluorescence microscope, the volume of the apoptotic cells decreased, with shrunken nuclei and a specific green fluorescence in the chromosome.

Flow cytometry

According to the literature [28], sub-G_1 peaks in untreated, curcumin derivative-treated and curcumin-treated cells were examined by flow cytometry. Briefly, 2×10^5 cells were collected, washed twice with 0.01 mol/l PBS and fixed in 70% ethanol overnight at 4°C. Then, cells were washed once with PBS, digested by 200 μl RNase (1 mg/ml) at 37°C for 30 min and stained with 800 μl propidium iodide (50 μg/ml) at room temperature for 30 min. The DNA histograms were assayed with flow cytometry (Becton Dickson, San Jose, California, USA), using CellQuest software.

Caspase-3 activity assay

Cells (2×10^5) from the above groups were collected, mixed with 50 μl cellular lysis buffer and then incubated on ice for 10 min. After centrifugation (12 000 r.p.m.) at 4°C for 3 min, the supernatant was collected and mixed sequentially with 50 μl 2 × reaction buffer and 5 μl caspase-3 substrate DEVD-pNA (1.0 mmol/l), and incubated at 37°C for 1 h. After being transferred into 96-well plates, the optical density values ($A_{405\,nm}$) of the slides were read on an enzyme-labeled Minireader II at a wavelength of 405 nm, which represented the intracellular activity of caspase-3.

Statistical analysis

Data were expressed as mean ± SD. The statistical significance of findings was determined via Student's *t*-test and ANOVA using SPSS 10.0 statistical software. $P < 0.05$ was considered significant.

Results

Structure determination of curcumin derivatives

The NVC and NGC synthetic derivatives of curcumin were obtained as brown–red powder. As determined by IR and NMR, as well as a comparison of spectral data with those for curcumin, the molecular formulas of these compounds were $C_{31}O_9H_{31}N$ and $C_{28}O_9H_{25}N$, respectively, consistent with the design and synthesis methods. ^{1}H-NMR indicated the chemical shifts and extent of stereospecific blockade (Table 1). On ^{13}C-NMR spectra, δ183.9 and δ185.7 stood for carbons within a carbonyl group; δ160.5 and δ181.9 for carbons within a carbonyl group at an ester linkage, while δ150.0, δ173.9 and δ171.2 for carbons within a carbonyl group of an endocyclic amide; δ148.7, δ141.4, δ140.0, δ150.2 and δ142.3 for aromatic carbons linked with OR; δ127.0 and δ139.5 for double-bonded carbons of an endocyclic amide; δ126.5, δ123.8, δ130.2, δ125.2, δ127.0 and δ134.8 for carbons at ethylenic linkages; δ131.0, δ112.0 and δ116.6 for aromatic carbons linked with branched chains; δ121.8, δ116.6, δ116.4, δ124.1, δ123.8, δ112.8 and δ122.8 for aromatic carbons; δ101.5 and δ101.2 for carbons linked with two carbonyls; δ50.3 and δ56.4 for methylenes linked with an ester group; and δ40.2 and δ46.2 for methylenes linked with carbonyls. All together, the signals of protons and carbons within these curcumin derivatives were readily

assigned, demonstrating that the *n*-maleoyl-L-valine and *n*-maleoyl-glycine groups had been covalently linked with curcumin via ester bonds.

Intracellular esterase-sensitive properties of curcumin derivatives

As shown in Fig. 2(a), the esterase activity within EJ cells was 10.2-fold higher than that of HKC cells ($P < 0.01$). In the presence of BNPP, a potent esterase inhibitor, the intracellular esterase activity of EJ and HKC cells was decreased by 65.63–96.9 ($P < 0.01$) and 46.9–88.7% ($P < 0.01$), in a dose-dependent manner. Administration of 20 μmol/l BNPP resulted in the lowest esterase activity within EJ and HKC cells. Moreover, pre-treatment of cells with 10 μmol/l BNPP abolished NVC- and NGC-induced cell viability inhibition in both EJ and HKC cells (Fig. 2b and c).

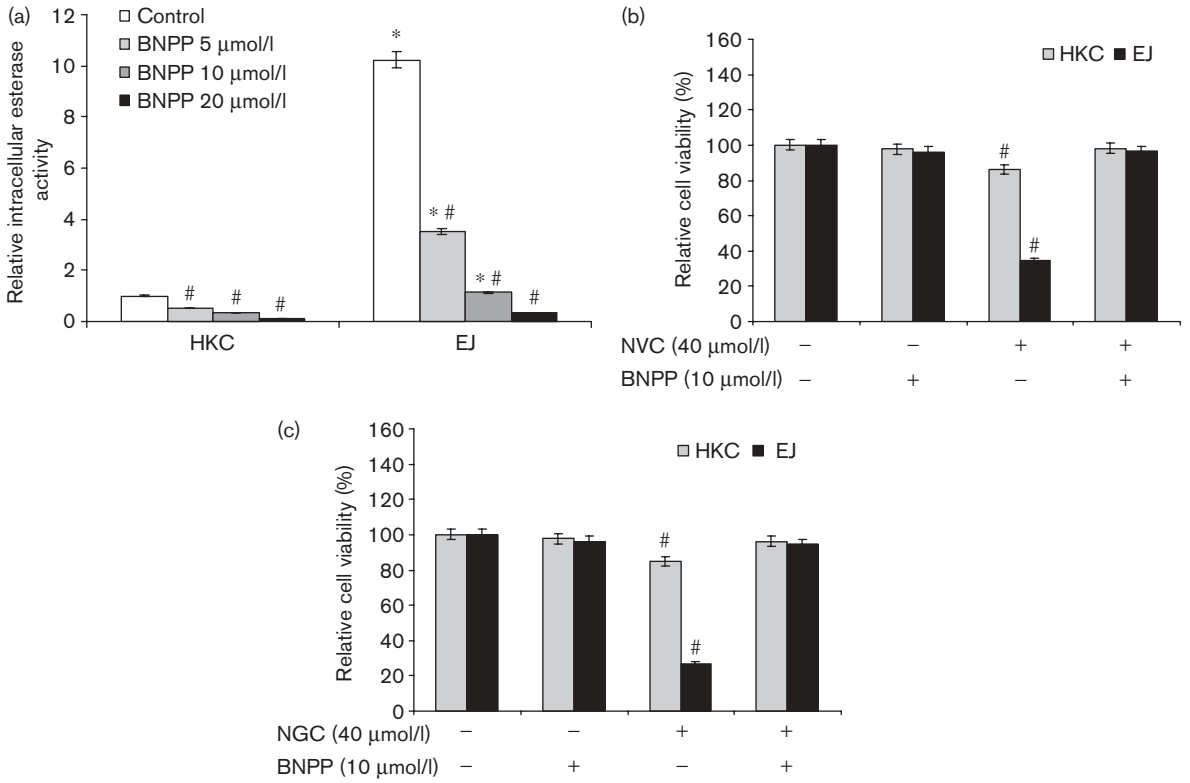
Table 1 ^{1}H-NMR analysis of NVC and NGC

NVC		NGC	
Chemical shift	No. of atoms	Chemical shift	No. of atoms
7.54–7.62	3	7.8	1
7.53–7.35	2	7.61–7.51	3
7.15–7.20	2	7.34–7.33	1
6.69–6.80	6	7.20–7.11	3
3.75–3.85	6	7.00–6.82	2
3.43–3.47	4	6.80–6.75	2
3.05–3.09	2	6.08–6.04	1
2.51	1	3.84–3.75	2
1.18–1.20	4	3.63–3.74	2
1.05–1.08	1	3.63–3.42	6
		3.15–3.07	2

Curcumin derivative-induced cell growth inhibition

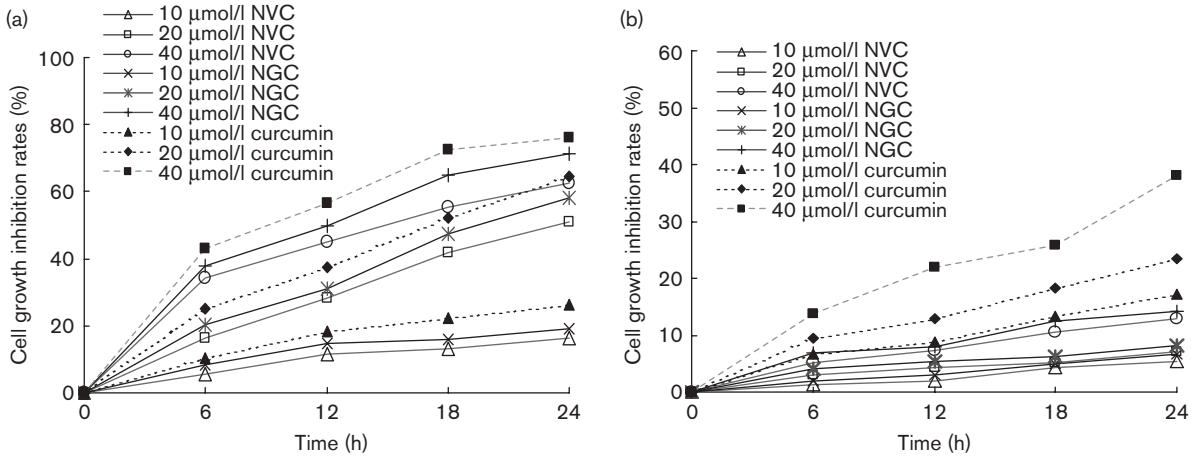
As shown in Fig. 3(a), treatment of EJ cells with 10–40 μmol/l NVC and NGC for 6–24 h resulted in growth inhibition by 5.63–62.65 ($P < 0.05$) and 8.28–71.22% ($P < 0.05$), respectively, in a time- and dose-dependent manner. The IC_{50} values of NVC and NGC on EJ cells were 20.1 and 18.7 μmol/l, respectively, which were close to that of curcumin (16.5 μmol/l). The growth inhibition effects of NGC were higher than those of NVC. Treatment of HKC cells with the same concentrations of these two derivatives resulted in growth inhibition by

Fig. 2



Intracellular esterase-sensitive properties of curcumin derivatives. Confluent monolayers of EJ and HKC cells were seeded into each well of 96-well plates, pre-treated with the esterase inhibitor BNPP as indicated and incubated with 40 μmol/l NVC or NGC for 24 h. Intracellular esterase activities and cell viability were examined by 6-CFDA fluorometry and MTT colorimetry, respectively. (a) The esterase activity within EJ cells was 10.2-fold higher than that of HKC cells. In the presence of BNPP, the esterase activity within both EJ and HKC cells was decreased. (b) Pre-treatment of cells with 10 μmol/l BNPP abolished NVC-induced cell viability inhibition in both EJ and HKC cells. (c) Similarly, pre-treatment of cells with 10 μmol/l BNPP abolished NGC-induced cell viability inhibition in both EJ and HKC cells. *Significant increase from HKC cells ($P < 0.05$). #Significant decrease from controls untreated with either BNPP or curcumin derivatives ($P < 0.05$). Triplicate experiments were performed with essentially identical results.

Fig. 3



Growth-inhibitory effects of curcumin derivatives on cancer and normal cells. Confluent monolayers of EJ and HKC cells were seeded into each well of 96-well plates, and treated with different concentrations of curcumin and its derivatives as indicated. Cell viability was monitored by MTT colorimetry. (a) Treatment of EJ cells with 10–40 μmol/l NVC and NGC for 6–24 h resulted in growth inhibition in a time- and dose-dependent manner. The IC_{50} values of NVC and NGC on EJ cells were 20.1 and 18.7 μmol/l, respectively, which was close to that of curcumin (16.5 μmol/l). (b) Treatment of HKC cells with these two derivatives resulted in decreased cell growth inhibition compared with curcumin. The IC_{50} values of NVC and NGC on HKC cells were 4.06- and 3.23-fold higher than that of curcumin. Triplicate experiments were performed with essentially identical results.

1.33–12.89 ($P < 0.05$) and 2.03–14.25% ($P < 0.05$), respectively, which were significantly lower than those of curcumin (6.77–38.08%, Fig. 3b). The IC_{50} values of NVC and NGC on HKC cells were 251.2 and 199.5 μmol/l, respectively, which were 4.06- and 3.23-fold higher than that of curcumin (61.8 μmol/l, $P < 0.01$).

Curcumin derivative-induced morphological changes of apoptosis

To investigate the type of cell death induced by curcumin derivatives, the cells were stained with AO/EB, which allows the identification of viable, apoptotic and necrotic cells based on color and appearance [27]. As shown in Fig. 4(a), treatment of EJ cells with 20 μmol/l NVC or NGC for 12 h resulted in shrunken nuclei and orange-stained cells, which had similar morphological changes to the curcumin treatment group. However, after treatment with 20 μmol/l curcumin derivatives for 12 h, there were no such morphological changes in HKC cells (Fig. 4b). This procedure was also used to quantify the number of apoptotic cells induced by curcumin derivatives. As shown in Tables 2 and 3, 10–40 μmol/l NVC and NGC exerted strong apoptosis-inducing effects on EJ cells rather than HKC cells. In addition, TUNEL labeling further indicated that treatment of EJ cells with 20 μmol/l NVC or NGC resulted in obvious DNA strand breakage, a characteristic change of apoptosis, which was similar to that of curcumin-treated cells (Fig. 4c and d).

Curcumin derivative-induced apoptosis rates

In order to quantify the apoptotic cells, the proportion of cells that had a DNA content of less than 2*N* (sub-G_1 DNA content) was measured by flow cytometry [28]. After treatment with 10–40 μmol/l NVC and NGC for 12 h, there were obvious sub-G_1 DNA peaks in EJ cells. The apoptosis rates were 10.13–23.36 ($P < 0.05$) and 12.42–28.56% ($P < 0.05$), respectively. Meanwhile, the apoptosis rates of HKC cells were 1.12–3.32 and 2.02–4.16%, which were significantly lower than those of curcumin-treated cells (4.12–11.32%) (Fig. 5).

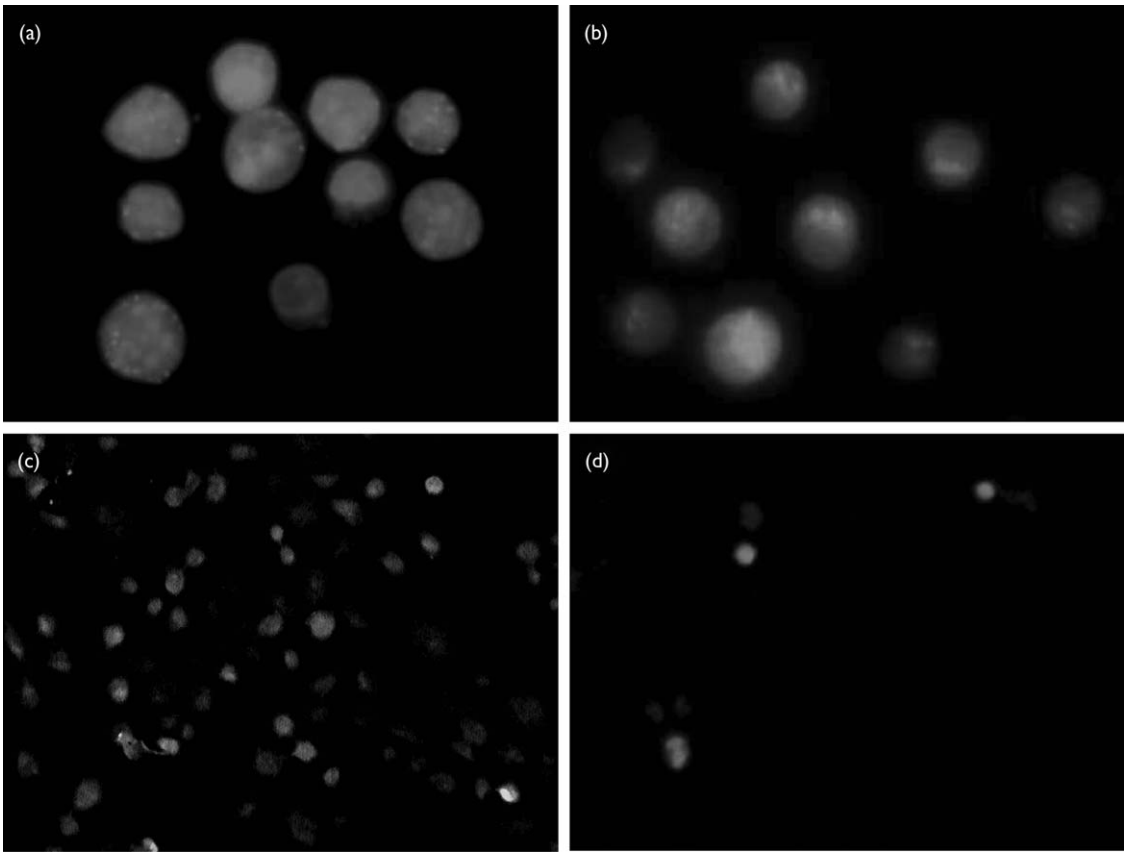
Curcumin derivative-induced caspase-3 activities

To observe the intracellular caspase-3 activity induced by curcumin derivatives, a spectrophotometric method was used. As shown in Fig. 6, following administration of 20 μmol/l NVC and NGC for 12 h, the caspase-3 activity of EJ cells was enhanced by 5.21- ($P < 0.01$) and 5.63-fold ($P < 0.01$), respectively, which was close to that induced by 20 μmol/l curcumin (6.42-fold). However, no significant increase of caspase-3 activity was observed in HKC cells after administration of these two curcumin derivatives ($P > 0.05$).

Discussion

The chemopreventive effects of curcumin have been attributed to various biological properties, including neutralization of carcinogenic free radicals [29], anti-angiogenesis action [30,31], inhibition of COX-1 and COX-2 enzymes, and stimulation of glutathione S-transferase [32]. Although the specific mechanism of its activity is not fully understood, this small molecule carotenoid pigment is a promising compound for structure modification. Studies on structure–activity relationship of curcuminoids have revealed that the β-diketone

Fig. 4



Apoptotic morphological changes induced by curcumin derivatives. Confluent monolayers of EJ and HKC cells were treated with 20 μmol/l of curcumin and its derivatives for 12 h. Cells were collected for AO/EB staining and TUNEL labeling. (a) AO/EB staining indicated that administration of NVC or NGC to EJ cells resulted in shrunken nuclei and orange-stained cells, which had similar morphological changes as the curcumin-treated group (× 400 original magnification). (b) After treatment with 20 μmol/l curcumin derivatives, there were no such morphological changes in HKC cells under fluorescence microscopy (× 400 original magnification). TUNEL labeling further indicated that administration of NVC and NGC in EJ cells (c), rather than HKC cells (d), resulted in extensive DNA strand breakage, a characteristic change of apoptosis, which was similar to that of curcumin-treated cells (× 100 original magnification). Triplicate experiments were performed with essentially identical results.

Table 2 Apoptosis of EJ cells detected by AO/EB fluorescent staining

Group	Cell number				Summation	Apoptosis rate (%)
	VA	NVA	NV	NVN		
Control	5	0	192	3	200	2.5
10 μmol/l NVC	10	6	178	6	200	8.0*
20 μmol/l NVC	16	22	142	14	200	19.0*
40 μmol/l NVC	20	41	120	19	200	30.5*
10 μmol/l NGC	12	10	170	8	200	11.0*
20 μmol/l NGC	17	28	138	17	200	22.5*
40 μmol/l NGC	21	48	111	20	200	34.5*
10 μmol/l curcumin	10	21	160	9	200	15.5*
20 μmol/l curcumin	19	39	121	21	200	27.5*
40 μmol/l curcumin	35	52	85	28	200	43.5*

*$P < 0.05$ compared with control group.

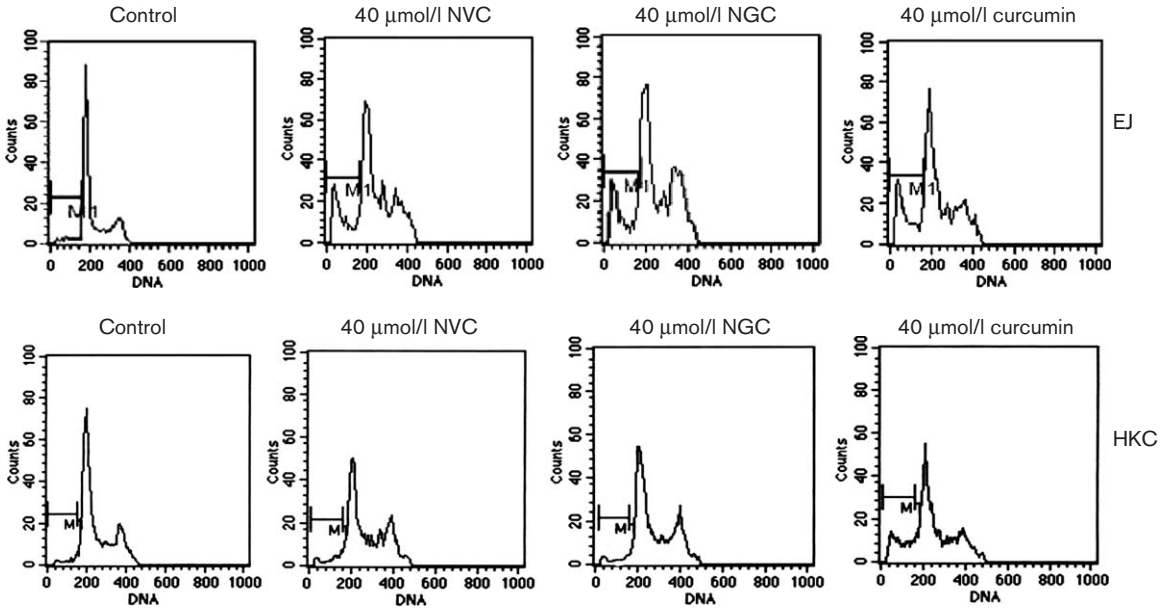
moiety of curcumin is essential for its anti-cancer activities [33]. In addition, phenolic hydroxyl or methoxyl groups were modified to enhance the potency of other biological activities of curcumin, including the induction of phase 2 detoxification enzymes and the inhibition of HIV-1 integrase [34,35]. A slight modification of the benzaldehyde moiety in the curcuminoid structure affected the inhibition of Fos–Jun and DNA complex formation [36]. Ishida *et al.* prepared 26 β-diketone compounds that were structurally related to curcumin

Table 3 Apoptosis of HKC cells detected by AO/EB fluorescent staining

Group	Cell number				Summation	Apoptosis rate (%)
	VA	NVA	NV	NVN		
Control	2	0	195	3	200	1.0
10 μmol/l NVC	3	0	194	3	200	1.5
20 μmol/l NVC	5	1	192	2	200	3.0
40 μmol/l NVC	7	5	185	3	200	6.0*
10 μmol/l NGC	4	0	194	2	200	2.0
20 μmol/l NGC	6	1	190	3	200	3.5
40 μmol/l NGC	10	3	184	3	200	6.5*
10 μmol/l curcumin	5	7	182	6	200	6.0*
20 μmol/l curcumin	8	13	171	8	200	10.5*
40 μmol/l curcumin	15	16	153	16	200	15.5*

*$P < 0.05$ compared with control group.

Fig. 5

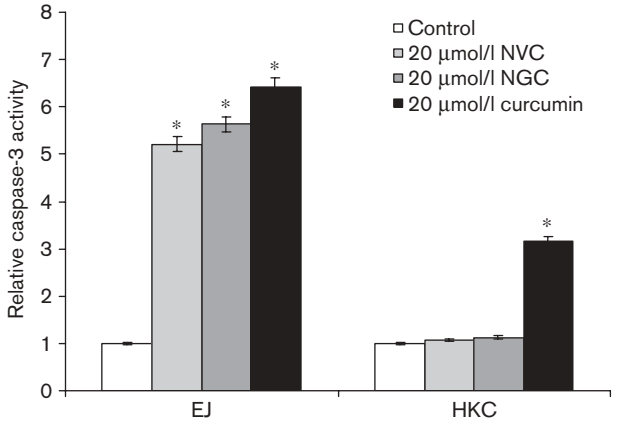


Flow cytometric assay of apoptosis induced by curcumin derivatives. Cells (2×10^5) were collected, washed twice with 0.01 mol/l PBS and fixed in 70% ethanol overnight at 4°C. Then, the cells were washed once with PBS, digested by 200 μl of RNase (1 mg/ml) at 37°C for 30 min and stained with 800 μl of propidium iodide (50 μg/ml) at room temperature for 30 min. The DNA histograms were assayed by flow cytometry using CellQuest software. The results indicated that administration of 40 μmol/l NVC and NGC resulted in obvious sub-G_1 DNA peaks in EJ cells, but not in HKC cells, which were similar to those of curcumin-treated cells. One representative experiment of three, all with similar results, is shown.

and evaluated their cytotoxic effects against a panel of human tumor cell lines [37]. They found that a curcumin derivative having an α-bromo substituent and 4-nitro and 4-methoxy groups on separate benzene rings demonstrated the strongest cytotoxic effects against HOS and 1A9 cells [37]. Also, the inhibitory action of a β-diketone derivative of curcumin on 7,12-dimethylbenz[*a*]anthracene-induced mammary tumorigenesis was reported [38]. In addition, curcumin derivatives showed potent inhibition of cell proliferation of MCF-7 human breast tumor cells [33]. Although many studies indicated that curcumin and its derivatives are not toxic even at high doses in laboratory animals, it still exerts cytotoxicity on normal cells [23,24]. Thus, new strategies are required to improve the selectivity of curcumin-mediated killing of tumor cells.

One approach to improving the selectivity of anti-cancer compounds is the use of their derivative forms that can be selectively activated in tumor tissue. Some researchers utilize unique aspects of tumor physiology, such as selective enzyme expression, hypoxia and/or low extracellular pH [39]. Since tumor cells have a more active metabolism than normal cells, with higher contents of intracellular phosphatase and esterase, this provides us with a potential way to modify anti-cancer drugs according to their structures and ameliorate their selectivity against tumor cells [39]. This is particularly true for the derivatization of carboxyl or hydroxyl groups to form ester functional groups, which are readily hydrolyzed in-vivo to release the parent drugs [39]. In the present study, we covalently linked the curcumin

Fig. 6



Caspase-3 activities induced by curcumin derivatives. Confluent monolayers of EJ and HKC cells were treated with 20 μmol/l of curcumin and its derivatives for 12 h. The intracellular caspase-3 activity was examined by spectrophotometric assay, using DEVD-pNA as substrate. The optical density values ($A_{405\,nm}$), indicating the intracellular activities of caspase-3, were read on an enzyme-labeled Minireader II at 405 nm. The results indicated that administration of NVC and NGC led to enhancement of caspase-3 activities in EJ cells, which were close to those induced by curcumin. However, no significant increase in caspase-3 activity was observed in HKC cells after administration of these two curcumin derivatives. *Significant increase from control cells untreated with curcumin and or its derivatives. Triplicate experiments were performed with essentially identical results.

with *n*-maleoyl-L-valine and *n*-maleoyl-glycine groups respectively, and developed two curcumin derivatives (NVC and NGC). The cell viability assay indicated that these curcumin derivatives effectively inhibited the in-vitro growth of bladder cancer EJ cells in a time- and dose-dependent manner, with similar IC_{50}s to curcumin. However, their IC_{50}s on HKC cells were 4.06- and 3.23-fold higher than curcumin. To explore their biological properties in these cell lines, we examined the intracellular esterase activity of EJ and HKC cells by 6-CFDA fluorometry. 6-CFDA is a fluorogenic substrate that can diffuse through cell surface membranes into the cytoplasm, where it is hydrolyzed by intracellular non-specific esterases to produce a green fluorescent product, carboxyfluorescein (CF) [25]. Being hydrophilic, CF is likely to be retained within the cell and detectable by fluorometry [25]. Using this method, we found that the bladder cancer EJ cells had 10.2-fold higher intracellular esterase activity than HKC cells. When the intracellular esterase activity was abolished by BNPP, an esterase inhibitor [26], the growth-inhibitory effects of these two curcumin derivatives were attenuated, which indicated their esterase-sensitive properties.

Previous reports have demonstrated that in many immortalized and cancer cell lines, such as c-*erbB2* oncogene-transformed NIH 3T3, mouse sarcoma S180, human colon cancer HT-29, human kidney cancer 293 and human hepatocellular carcinoma HepG2 cells, curcumin could induce characteristics of apoptosis such as cell shrinkage, chromatin condensation and DNA fragmentation. Our previous data have indicated that curcumin could induce apoptosis of cancer cells through blocking the function of NF-κB and regulating expression of apoptosis-associated genes (p53, Bcl-2, MDM2) [40]. In this study, administration of curcumin derivatives NVC and NGC to EJ cells resulted in characteristic apoptotic changes as detected by AO/EB staining and TUNEL labeling. The results from flow cytometry indicated that after administration of 40 μmol/l NVC and NGC for 12 h, apoptotic rates of EJ cells reached 23.36 and 28.56%, respectively. However, due to the low intracellular esterase content, treatment of HKC cells with these curcumin derivatives resulted in lower growth inhibition and apoptosis-inducing effects when compared with curcumin at the same concentrations. Moreover, our data indicated that NVC and NGC enhanced caspase-3 activity in EJ cells, but not HKC cells, which was consistent with recent reports that augmentation of caspase-3 activity was involved in curcumin-induced apoptosis of cancer cells [41]. The exact signal transduction pathways involved in curcumin derivative-induced apoptosis and caspase-3 activation warrants further study.

In summary, two curcumin derivatives were synthesized and showed promising chemopreventive activities, although none of the compounds was significantly more potent than curcumin in these assays. Their cytotoxicity on normal cells was attenuated when compared to curcumin. Further studies need to be carried out to clarify their in-vivo bioavailability and chemopreventive properties on animal models.

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