Synthesis and Evaluation of Curcumin-Related Compounds Containing Benzyl Piperidone for Their Effects on Human Cancer Cells

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Eleven curcumin-related compounds containing a benzyl piperidone moiety were synthesized and evaluated for their effects on cultured prostate cancer PC-3 cells, pancreas cancer BxPC-3 cells, colon cancer HT-29 cells and lung cancer H1299 cells. Inhibitory effects of these compounds on the growth of PC-3, BxPC-3, HT-29 and H1299 cells were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and trypan blue exclusion assay. Compounds benzyl piperidone 2 (P2), P4, P7, 4-bromo-2-fluoro-benzyl piperidone 2 (PFBr2), PFBr3 and PFBr4 (see syntheses and structures in Figs. 1, 2) exhibited potent inhibitory effects on the growth of cultured PC-3, BxPC-3, HT-29 and H1299 cells. The IC_{50} for these compounds was lower than 2 μM in all four cell lines. PFBr4 was 41-, 36-, 40- and 46-fold more active than curcumin for inhibiting the growth of PC-3, BxPC-3, HT-29 and H1299 cells, respectively. The benzyl piperidone-containing compounds studied also stimulated apoptosis in PC-3 cells. Mechanistic studies indicate that the effects of both curcumin and PFBr4 on PC-3 cells were associated with a decrease in phospho-Akt and phospho-extracellular signal-regulated kinase (Erk)1/2. The present study indicates that P2, P4, P7, PFBr2, PFBr3 and PFBr4 may have useful effects on human cancer cells.

Key words cancer cell; benzyl piperidone; curcumin-related compound; apoptosis

Curcumin is a yellow compound isolated from the dried rhizomes of the plant *Curcuma longa*, and is used for traditional medicine in India and China.[1)] Numerous studies have shown that curcumin possesses multifunctional pharmacological properties including anti-oxidant activity,[2–4)] anti-inflammatory activity[5–7)] and activity.[8–12)] Its activity is related in part to its ability to induce apoptosis in cancer cells. Because of its and antiangiogenic properties, low molecular weight and lack of toxicity, curcumin is under study as a possible new drug.[13)]

Curcumin has been evaluated in clinical trials for the treatment of liver disease, rheumatoid arthritis, infectious diseases and cancer. Despite its safety, the clinical usefulness of curcumin is diminished by extensive first-pass metabolism, resulting in low oral bioavailability.[14–16)] In the last decade curcumin has been much explored and various synthetic analogues have been prepared and evaluated for various pharmacological activities.[17–26)] Some analogues have shown good activities in various models and various cell lines. A recent study demonstrated that curcumin-related compounds with a benzyl piperidone had enhanced absorption and biological activities.[27,28)] The IC_{50} of these compounds in different cancer cell lines ranged from 5.5–8.3 μM.

In the present study, eleven curcumin-related compounds using benzyl piperidone (P) or 4-bromo-2-fluoro-benzyl piperidone (PFBr) as a linker and various substituents on the aryl rings (Fig. 1) were synthesized and evaluated for their effects against four human cancer cell lines. PFBr1, PFBr2, PFBr3 and PFBr4 are novel compounds with fluorine and bromine. P1 to P7 are known compounds.[27–32)] The curcumin-related compounds containing benzyl piperidone were evaluated in the present study and they showed a potent effect for inhibiting the growth of PC-3, BxPC-3, HT-29 and H1299 cells and for stimulating apoptosis in PC-3 cells. We also discuss the structure–activity relationship of curcumin-related compounds with a benzyl piperidone.

Results and Discussion

Chemistry A series of curcumin-related compounds containing benzyl piperidone were synthesized by coupling the appropriate substituted benzaldehyde with P or PFBr (Fig. 1). The synthesis and characterization of PFBr1, PFBr2, PFBr3 and PFBr4 were not previously reported. The synthesis and characterization of P1 to P7 are known, but no report on the activity on these compounds was found. Structures of curcumin and curcumin-related compounds containing a benzyl piperidone moiety are shown in Fig. 2.

Inhibitory Effects of Curcumin-Related Compounds Containing Benzyl Piperidone toward Cultured Human Prostate, Pancreas, Colon and Lung Cancer Cells The inhibitory effects of eleven curcumin-related compounds containing benzyl piperidone on the growth of cultured prostate cancer PC-3 cells, pancreas cancer BxPC-3 cells, colon cancer HT-29 cells and lung cancer cells H1299 were determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the trypan blue exclusion assay. For the experiments using the MTT assay, curcumin was evaluated as a positive control in each incubation. The inhibitory effect of curcumin did not vary significantly between the different incubations. Data from all curcumin incubations in experiments with group P and group PFBr compounds were averaged and presented in Table 1.

All compounds had stronger inhibitory effects than curcumin as determined by the MTT assay. Among the eleven curcumin-related compounds tested, PFBr4 exhibited excep-

The authors declare no conflict of interest.

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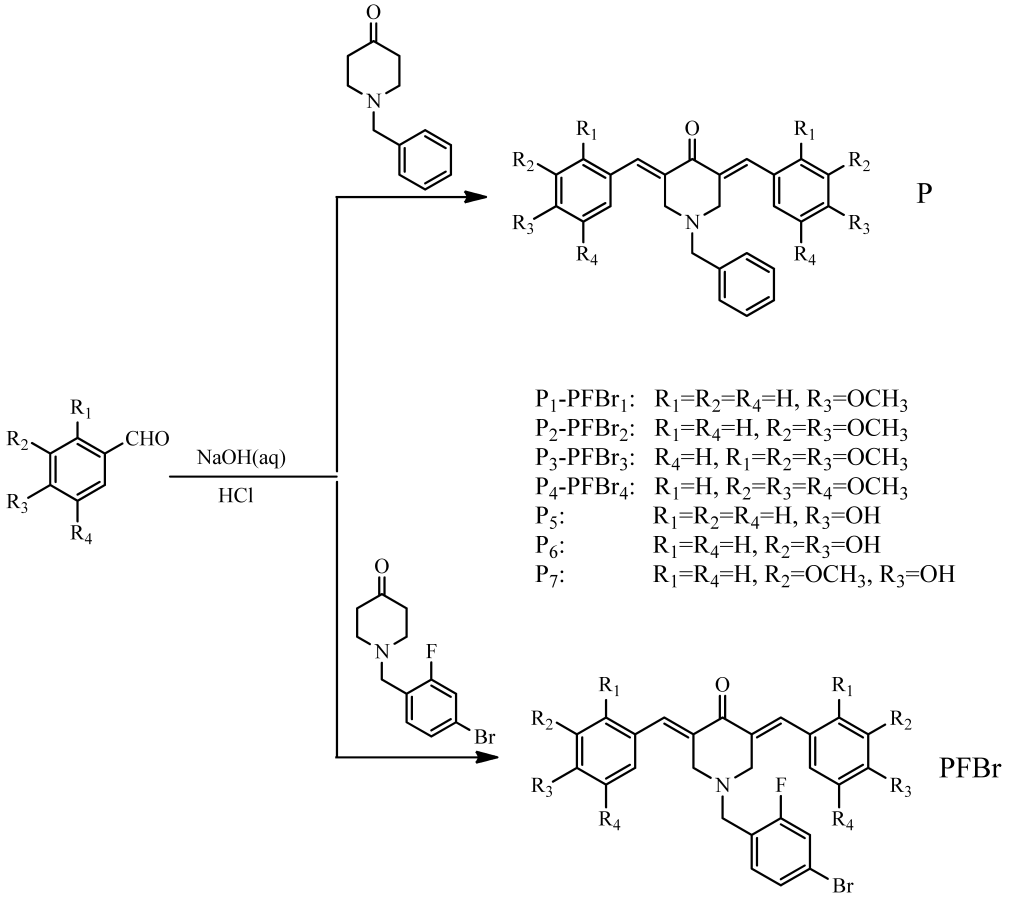


Fig. 1. Synthesis of Group P and Group PFBr Compounds

tionally potent inhibitory effects on the growth of cultured PC-3, BxPC-3, HT-29 and H1299 cells. The IC_{50} for this compound was $\leq 0.5\,\mu$M in all four cell lines, indicating that PFBr4 was approximately 40-fold more active than curcumin. As shown in Table 1, the IC_{50} values of all eleven curcumin-related compounds ranged from 0.41 to 17.67 μM in the four cell lines studied.

Because the achievable concentration of curcumin and curcumin-related compounds *in vivo* may be low, we further determined the effects of these compounds at 1 μM on the grown of PC-3 cells by using the trypan blue exclusion assay. As shown in Table 2, compound-related decreases in the number of viable cells were observed. Compared to the control group, the number of viable cells decreased by 14.7 to 99.3% when the cells were treated with curcumin and curcumin-related compounds (Table 2). The effects of curcumin and curcumin-related compounds at 1 μM on apoptosis of PC-3 cells were determined by morphological assessment of propidium iodide stained cells. In these experiments, PC-3 cells were treated with curcumin and curcumin-related compounds for 96 h. As shown in Fig. 3, weak stimulatory effects on the induction of apoptosis were observed in PC-3 cells treated with curcumin (1 μM), while much more obvious effects were seen in PC-3 cells treated with PFBr4 (1 μM). An earlier study showed that curcumin-related compounds with a benzyl piperidone had enhanced cytotoxic potential in colon, pancreas, lung and renal cancer cells.[27] In the present study, we found that compounds with similar structure had even stronger cytotoxic effects in prostate, pancreas, colon and lung cancer cells. Differences in cytotoxic effects between our compounds and those reported in the earlier study may be due to different cell lines used in each study. A previous study also indicated that piperidone enhanced the absorption of curcumin.[28] Although we did not monitor the uptake of the curcumin-related compounds containing benzyl piperidone in the present study, it is possible that enhancement of absorption contribute to increased activities of these compounds. Further studies are needed to determine the cellular uptake of these compounds.

To further determine the mechanisms for growth inhibition and apoptosis induction in PC-3 cells, we used a nuclear factor kappa B (NF-κB)-luciferase reporter gene expression assay to determine the effect of curcumin-related compounds (IC_{50} $<2\,\mu$M) on activation of NF-κB. In these experiments, VCaP/N cells were treated with a low concentration (1 μM) of curcumin and curcumin-related compounds for 24 h. Treatment of VCaP/N cells with group PFBr4 compounds resulted in strong decreases in NF-κB transcriptional activity (Fig. 4). Compounds P2, P4, P7, PFBr2 and PFBr3 had moderate effects for decreasing NF-κB transcriptional activity while curcumin had no effect (Fig. 4).

The levels of activated Akt and extracellular signal-regulated kinase (Erk)1/2 were evaluated by Western blot analysis using anti-phosphorylated Akt and anti-phosphorylated Erk1/2 antibodies (Fig. 5). In these experiments, PC-3 cells were treated with curcumin (1, 20 μM) or with PFBr4 (0.5, 1 μM) for 24 h and analyzed by Western blotting. The levels of phosphorylated Akt and Erk1/2 in Western blots were analyzed by optical density measurements and normalized for β-actin. As

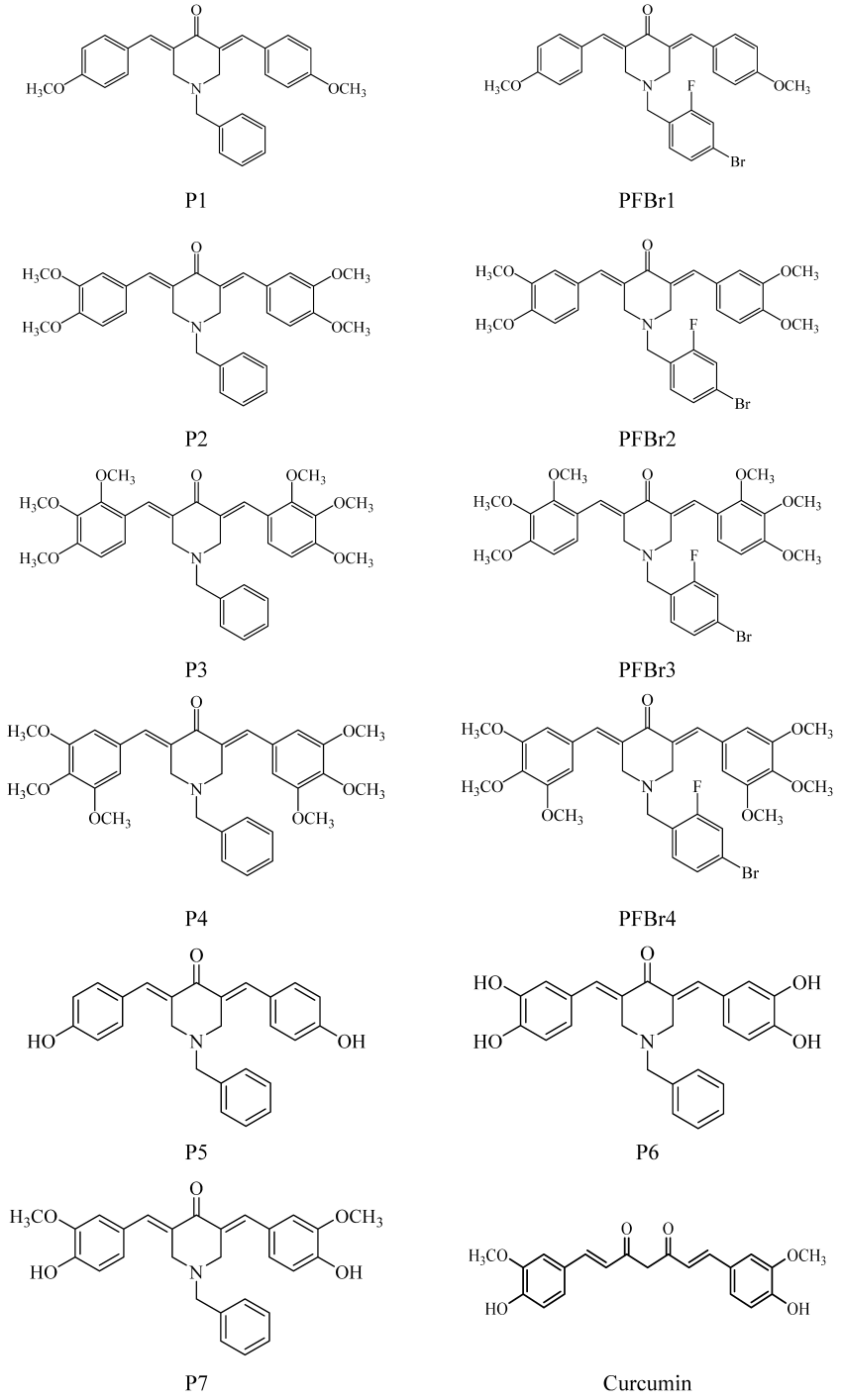


Fig. 2. Structures of Curcumin and Curcumin-Related Compounds

shown in Fig. 5, treatment of PC-3 cells with PFBr4 resulted in a strong decrease in the level of phosphorylated Erk1/2 while curcumin was inactive. Treatment with curcumin and PFBr4 all caused a decrease in the level of phosphorylated Akt. Our results indicate that the effects of both curcumin and PFBr4 on PC-3 cells were associated with a decrease in phospho-Akt and phospho-Erk1/2.

Structure–Activity Relationship Earlier studies on the analysis of the relationship between the structures of curcumin-related compounds and their ability to inhibit the growth of cultured cancer cells showed that the linker, the aromatic rings and steric hinderance are all very important for activity.[33] In the present study, we showed that using benzyl piperidone as a linker enhanced the cytotoxic potential of the curcumin-related compounds. We also found that PFBr3 and PFBr4 had huge increases in activity as compared to P3 and P4, indicating that the fluorine group in the benzyl piperidone linker conferred improvement in activity in these compounds. Structure–activity relationship analysis for the aromatic rings showed that reactive groups such as methoxy can enhance activity. P4 and PFBr4 showed some increases in activities over the corresponding P1 and PFBr1. P5 had slightly less activity than the corresponding P7. For the same linker and same aromatic rings, such as P3-P4 and PFBr3-PFBr4, steric

Table 1. Inhibitory Effects of Curcumin-Related Compounds Containing Benzyl Piperidone on the Growth of PC-3, BxPC-3, HT-29 and H1299 Cells

Compound	IC_{50} (μM)			
	PC-3	BxPC-3	HT-29	H1299
Curcumin	19.98±2.4	18.25±2.2	18.74±2.2	18.93±2.1
P1	9.13±1.3	6.67±0.9	7.95±1.1	5.61±0.7
P2	1.35±0.2	0.91±0.1	0.86±0.1	1.05±0.2
P3	10.18±2.1	8.93±1.2	9.16±1.2	10.95±1.6
P4	1.75±0.2	1.13±0.2	1.37±0.3	1.45±0.2
P5	5.05±0.7	2.64±0.3	4.97±0.6	1.58±0.2
P6	4.12±0.6	1.97±0.3	2.79±0.4	2.01±0.3
P7	1.82±0.3	1.09±0.2	1.55±0.2	1.31±0.2
PFBr1	17.67±2.6	15.59±2.4	16.43±2.1	17.23±2.7
PFBr2	1.78±0.2	1.70±0.2	2.38±0.4	2.16±0.3
PFBr3	1.75±0.2	1.51±0.2	1.63±0.3	2.59±0.4
PFBr4	0.49±0.1	0.50±0.1	0.47±0.1	0.41±0.1

Human prostate cancer PC-3, pancreas cancer BxPC-3, colon cancer HT-29 and lung cancer cells H1299 were seeded at a density of 0.2×10^5 cells/mL of medium in 96-well plates (0.2 mL/well) and incubated for 24 h. The cells were then treated with various concentrations (0.1–30 μM) of the different compounds for 72 h. Effects of the different compounds on the growth of PC-3, BxPC-3, HT-29 and H1299 cells were determined by the MTT assay. Each value is the mean±S.E. from three separate experiments.

Table 2. Effects of Curcumin-Related Compounds Containing Benzyl Piperidone on the Growth of PC-3 Cells

Treatment	No. of viable cells (1×10^{-4})	% growth inhibition
Control	44.2±2.7	—
Curcumin	37.7±2.4	14.7
P1	27.8±1.7	37.1
P2	13.2±1.5	70.1
P3	28.9±2.1	34.6
P4	14.5±1.2	67.2
P5	28.7±2.3	35.1
P6	26.2±2.0	40.7
P7	24.9±1.8	43.7
PFBr1	34.3±2.5	22.4
PFBr2	10.6±1.2	76.0
PFBr3	13.9±1.4	68.6
PFBr4	0.3±0.1	99.3

Human prostate cancer PC-3 cells were seeded at a density of 0.2×10^5 cells/mL in 35-mm tissue culture dishes and incubated for 24 h. The cells were then treated with curcumin and curcumin-related compounds (all at 1 μM) for 96 h. The number of viable cells was determined by the trypan blue exclusion assay. Each value represents the mean±S.E. from three separate experiments.

hinderance is very important for activity; P4 had increased activity as compared to P3, and PFBr3 was slightly less active than PFBr4.

Experimental

General Procedures Melting points were determined on a Yanagimoto micro melting apparatus and were uncorrected. The ^{1}H-NMR spectra were measured on a Varian Gemini-2000 spectrometer using dimethyl sulfoxide (DMSO) as solvent unless otherwise specified. Chemical shifts for ^{1}H-NMR (300 MHz) and ^{13}C-NMR (75 MHz) were expressed in ppm units with tetramethylsilane (TMS) as an internal standard. Multiplicities were recorded as s (singlet), br s (broad singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). Mass spectra were obtained on an LC-MS-2010A spectrometer with electrospray ionization (ESI). Elemental analyses were performed on an elemental analyser (Vario El). Thin-layer chromatography (TLC) was performed on Merck silica gel plates (DC-60 F254). Curcumin was isolated from the extract of *Curcuma longa* Linn according to a previous report.[34] All reagents (highest grade) were used as received unless otherwise stated.

Synthesis of Curcumin Analogues A total of 11 curcumin-related compounds containing benzyl piperidone were synthesized as previously described with modification.[34,35] A mixture of the appropriate aldehyde (0.01 mol) and the ketone (0.005 mol) was dissolved in glacial acetic acid saturated with anhydrous hydrogen chloride and heated in a water bath at 25–30°C for 2 h. After standing for 2 d, the mixture was treated with cold water and filtered. The solid obtained was then washed and dried. The crude product was recrystallized from appropriate solvents (methanol or ethanol). PFBr1 to PFBr4 are novel compounds and were not reported. P1 to P7 were known compounds.

1-Benzyl-3,5-bis(4-methoxyphenyl methylene)piperidin-4-one (P1): Yield 90%. mp 148–150°C. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.80 (s, 2H, CH=), 7.34 (d, J=8.0 Hz, 4H, ArH), 7.32–7.29 (m, 5H, ArH), 6.92 (d, J=8.0 Hz, 4H, ArH), 3.87 (s, 4H, –CH_2–N–CH_2–), 3.84 (s, 6H, –OCH_3), 3.75 (s, 2H, –CH_2–). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): 54.5, 55.3, 61.5, 114.0, 127.3, 128.0, 128.3, 129.0, 131.5, 132.2, 136.0, 137.5, 160.2, 187.6. ESI-MS (m/z): 426 $(M+1)^+$. $C_{28}H_{27}NO_3$: 425.

1-Benzyl-3,5-di(3,4-dimethoxybenzene methylene)piperidin-4-one (P2): Yield 92%. mp 168–170°C. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.75 (s, 2H, –CH=), 7.27–7.22 (m, 5H, ArH), 6.96 (d, J=8.1 Hz, 2H, ArH), 6.86 (s, 2H, ArH), 6.84 (d, J=8.9 Hz, 2H, ArH), 3.87 (s, 12H, –OCH_3), 3.85 (s, 4H, –N–CH_2–), 3.73 (s, 2H, –CH_2–). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): 54.4, 55.8, 55.9, 61.8, 111.0, 113.4, 124.2, 127.4, 128.2, 128.3, 129.0, 131.6, 136.2, 137.3, 148.7, 179.9, 187.3. ESI-MS (m/z): 486 $(M+1)^+$. $C_{30}H_{31}NO_5$: 485.

1-Benzyl-3,5-di(2,3,4-trimethoxybenzaldehyde methylene)-piperidin-4-one (P3): Yield 89%. mp 166–168°C. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 8.02 (s, 2H, –CH=), 7.22 (d, J=8.5 Hz, 5H, ArH), 6.87 (d, J=8.7 Hz, 2H, ArH), 6.64 (d, J=8.7 Hz, 2H, ArH), 3.89 (s, 18H, –OCH_3), 3.80 (s, 4H, –CH_2–N), 3.68 (s, 2H, –CH_2–). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): 54.4, 56.0, 60.8, 60.9, 61.5, 106.8, 122.4, 125.1, 127.1, 128.2, 128.9, 132.0, 132.4, 137.6, 142.3, 153.5, 154.6, 187.5. ESI-MS (m/z): 546 $(M+1)^+$. $C_{32}H_{35}NO_7$: 545.

1-Benzyl-3,5-di(3,4,5-trimethoxybenzaldehyde methylene)-piperidin-4-one (P4): Yield 78%. mp 140–142°C. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.64 (s, 2H, –CH=), 7.23–7.27 (m, 5H, ArH), 6.50 (s, 4H, ArH), 3.86 (s, 18H, –OCH_3), 3.84 (s, 4H, –CH_2–N–), 3.80 (s, 2H, –CH_2–). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): 54.2, 56.0, 60.8, 61.8, 103.6, 107.8, 127.5, 128.3, 129.1, 130.6, 132.5, 136.5, 136.8, 139.0, 152.9, 153.1, 187.0. ESI-MS (m/z): 546 $(M+1)^+$. $C_{32}H_{35}NO_7$: 545.

1-Benzyl-3,5-bis(4-dihydroxyphenyl methylene)piperidin-4-one (P5): Yield 53%. mp 200–202°C. ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 9.87 (s, 2H, Ar-OH), 7.81 (s, 2H, –CH=), 7.65 (d, J=8.0 Hz, 2H, 2×ArH), 7.37 (d, J=8.0 Hz, 2H, ArH), 7.32–7.37 (m, 5H, ArH), 6.88 (m, 4H, ArH), 4.55 (s, 6H, –CH_2–N–). ESI-MS (m/z): 398 $(M+1)^+$. $C_{26}H_{23}NO_3$: 397.

1-Benzyl-3,5-di(3,4-dihydroxyphenyl methylene)piperidin-

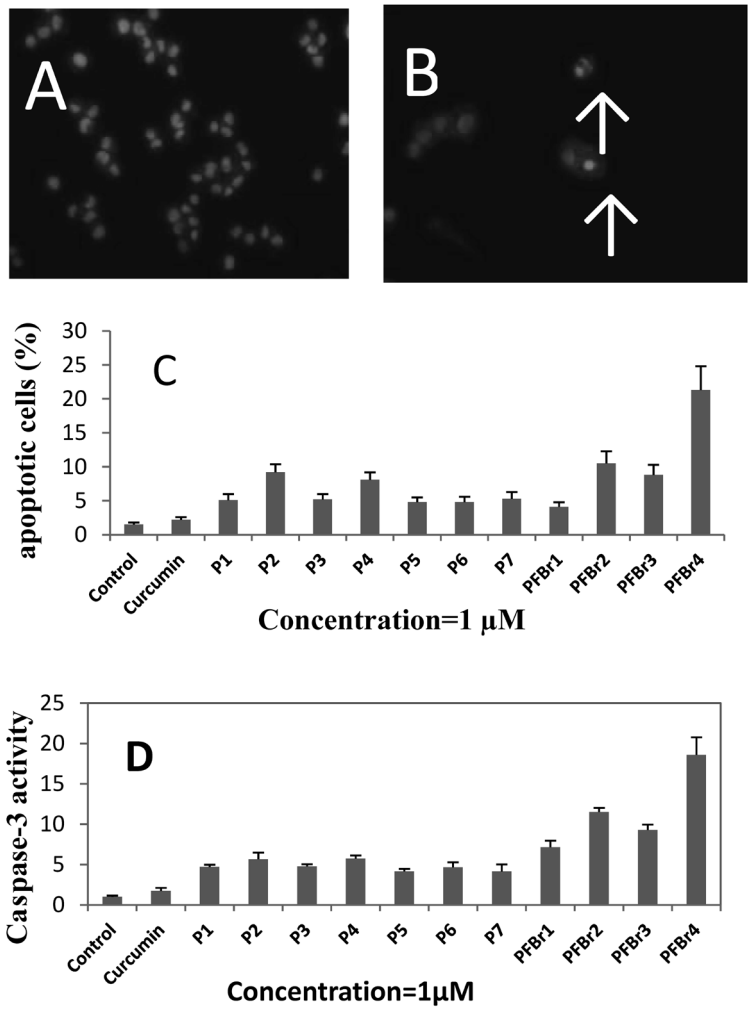


Fig. 3. Effects of Curcumin-Related Compounds Containing Benzyl Piperidone on Apoptosis of PC-3 Cells

Human prostate cancer PC-3 cells were seeded at a density of 0.2×10^5 cells/mL in 35-mm tissue culture dishes and incubated for 24 h. The cells were then treated with curcumin and curcumin-related compounds (all at 1 μM) for 96 h. (A, B) Representative micrographs of propidium iodide-stained controls and PFBr4 treated PC-3 cells. (C) Percentage of apoptotic cells as determined by morphological assessment in PC-3 cells treated with curcumin and curcumin-related compounds. (D) Caspase-3 activities in PC-3 cells treated with curcumin and curcumin-related compounds. Each value represents the mean±S.E. from three separate experiments.

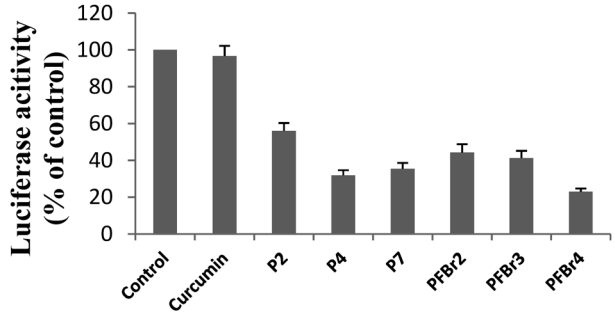
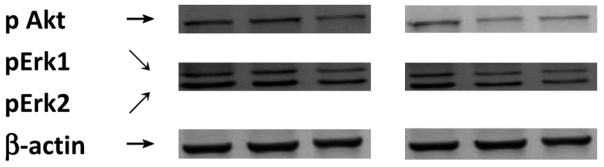


Fig. 4. Inhibitory Effects of Curcumin-Related Compounds Containing Benzyl Piperidone on NF-κB Transcriptional Activity in VCaP/N Cells

VCaP/N cells were seeded at a density of 0.2×10^5 cells/mL of medium in 24-well plates (1.0 mL/well) and incubated for 24 h. The cells were then treated with 1 μM of the different compounds for 24 h. The NF-κB transcriptional activity was determined by the luciferase reporter assay. Each value represents the mean±S.E. from three separate experiments.



	DMSO	CUR 1μM	PFBr4 0.5μM	DMSO	CUR 20μM	PFBr4 1μM
p Akt	1.00	1.03	0.48	1.00	0.49	0.51
pErk1	1.00	1.12	0.75	1.00	0.72	0.51
pErk2	1.00	1.02	0.68	1.00	0.79	0.48

Fig. 5. Effects of Curcumin and PFBr4 on the Activation of Akt and Erk1/2 in PC-3 Cells

PC-3 cells were seeded at a density of 1×10^5 cells/mL of medium in 100 mm culture dishes (10 mL/dish) and incubated for 24 h. The cells were then treated with DMSO (final concentration 0.01%) curcumin (CUR; 1, 20 μM in DMSO final concentration 0.01%), PFBr4 (0.5, 1 μM in DMSO final concentration 0.01%) for 24 h. Activated Akt and Erk1/2 were determined by using Western blot analysis with anti-phosphorylated-Akt or anti-phosphorylated-Erk1/2 antibody. The extent of protein loading was determined by blotting for β-actin and the levels of phosphorylated Akt and Erk1/2 in Western blots were analyzed by optical density measurements and normalized for β-actin to obtain the relative optical densities.

4-one (P6): Yield 51%. mp 232–234°C. ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 7.73 (s, 2H, –CH=), 7.56 (d, J=6.4, 2H, ArH), 7.40–7.29 (m, 3H, ArH), 6.89 (m, 4H, ArH), 6.80 (d, 2H, J=8.4 Hz, ArH), 4.52 (s, 6H, –CH_2–N). ESI-MS (m/z): 430 $(M+1)^+$. $C_{26}H_{23}NO_5$: 429.

1-Benzyl-3,5-bis(4-hydroxy-3-methoxybenzoate methylene)-piperidin-4-one (P7): Yield 66%. mp 210–212°C. ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 9.87 (m, 2H, Ar-OH), 7.81 (s, 2H, ArH), 7.58 (s, 2H, ArH), 7.39 (d, J=4.1, 3H, ArH), 7.04 (m, 6H, ArH), 4.58 (s, 6H, –CH_2–N–), 3.83 (s, 4H, –OCH_3). ESI-MS (m/z): 458 $(M+1)^+$. $C_{26}H_{23}NO_3$: 457.

1-(4-Bromo-2-fluoro-benzyl)-3,5-bis(4-methoxybenzene methylene)piperidin-4-one (PFBr1): Yield 91%. mp 170–172°C. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.80 (s, 2H, –CH=), 7.34 (d, J=8.5 Hz, 4H, ArH), 7.22–7.17 (m, 3H, ArH), 6.94 (d, J=8.6 Hz, 4H, ArH), 3.88 (s, 4H, –CH_2–N–), 3.86 (s, 6H, –OCH_3), 3.76 (s, 2H, –CH_2–). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): 53.5, 54.4, 55.5, 111.1, 118.8, 119.0, 121.3, 123.5, 127.4, 127.8, 131.1, 132.3, 136.3, 160.3, 162.2, 187.2. ESI-MS (m/z): 523 $(M+1)^+$. $C_{28}H_{25}BrFNO_3$: 522.

1-(4-Bromo-2-fluoro-benzyl)-3,5-bis(3,4-methoxybenzenemethylene)piperidin-4-one (PFBr2): Yield 93%. mp 211–213°C. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.74 (s, 2H, –CH=), 7.17 (s, 3H, ArH), 6.92 (d, J=16.0 Hz, 6H, ArH), 3.91 (s, 4H, –CH_2–N), 3.87 (s, 12H, –OCH_3), 3.76 (s, 2H, –CH_2–). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): 53.6, 54.3, 55.8, 55.9, 111.0, 115.5, 118.7, 121.4, 123.0, 124.1, 127.4, 128.1, 131.2, 132.5, 136.5, 148.8, 150.0, 162.2, 186.8. ESI-MS (m/z): 583 $(M+1)^+$. $C_{30}H_{29}BrFNO_5$: 582.

1-(4-Bromo-2-fluoro-benzyl)-3,5-bis(2,3,4-methoxybenzene methylene)piperidin-4-one (PFBr3): Yield 82%. mp 151–153°C. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 8.01 (s, 2H, –CH=), 7.16–7.12 (m, 3H, ArH), 6.87 (d, J=8.5 Hz, 2H, ArH), 6.66 (d, J=8.6 Hz, 2H, ArH), 3.90 (s, 18H, –OCH_3), 3.79 (s, 4H, –CH_2–N–CH_2–), 3.69 (s, 2H, –CH_2–). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): 52.9, 54.4, 56.0, 60.9, 61.5, 106.8, 118.6, 118.9, 122.2, 123.6, 123.7, 125.1, 127.2, 132.1, 132.2, 142.3, 153.5, 154.8, 159.7, 187.0. ESI-MS (m/z): 643 $(M+1)^+$. $C_{32}H_{33}BrFNO_7$: 642.

1-(4-Bromo-2-fluoro-benzyl)-3,5-bis(3,4,5-methoxybenzene methylene)piperidin-4-one (PFBr4): Yield 83%. mp 169–172°C. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.70 (s, 2H, –CH=), 7.18 (s, 3H, ArH), 6.59 (s, 4H, ArH), 3.88 (s, 4H, –CH_2–N–CH_2–), 3.87 (s, 18H, –OCH_3), 3.78 (s, 2H, –CH_2–). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): 53.8, 54.2, 56.1, 60.9, 107.8, 118.8, 119.0, 122.5, 122.6, 127.5, 130.5, 132.2, 136.6, 139.1, 153.1, 159.8, 186.7. ESI-MS (m/z): 643 $(M+1)^+$. $C_{32}H_{33}BrFNO_7$: 642.

Cell Culture and Reagents PC-3, BxPC-3, HT-29 and H1299 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, U.S.A.). RPMI-1640 tissue culture medium, penicillin–streptomycin, L-glutamine and fetal bovine serum (FBS) were from Gibco (Grand Island, NY, U.S.A.). The cells were maintained in RPMI-1640 culture medium, which were supplemented with 10% FBS, penicillin (100 units/mL)–streptomycin (100 μg/mL) and L-glutamine (300 μg/mL). Cultured cells were grown at 37°C in a humidified atmosphere of 5% CO_2 and were passaged twice a week. Curcumin-related compounds were dissolved in DMSO and the final concentration of DMSO in all experiments was 0.1%.

MTT, Trypan Blue Assays PC-3, BxPC-3, HT-29 and H1299 cells were seeded at a density of 0.2×10^5 cells/mL of medium in 96-well plate (0.2 mL/well) and incubated for 24 h. The cells were then treated with various concentrations (0.2–20 μM) of curcumin-related compounds containing benzyl piperidone for 72 h. After treatment, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide was added to each well of the plate and incubated for 1 h. After careful removal of the medium, 0.1 mL DMSO was added to each well, and absorbance at 550 nm was recorded on a microplate reader. The number of viable cells after each treatment was determined using a hemacytometer under a light microscope (Nikon Optiphot, Japan). Cell viability was determined by the trypan blue exclusion assay, which was done by mixing 80 μL of cell suspension and 20 μL of 0.4% trypan blue stain solution for 2 min. Blue cells were counted as dead cells and the cells that did not absorb dye were counted as live cells.

Assessment of Apoptotic Cells by Morphology and Activation of Caspase-3 Apoptotic cells were determined by morphological assessment in cells stained with propidium iodide. Cytospin slides were prepared after each experiment and cells were fixed with acetone–methanol (1 : 1) for 10 min at room temperature, followed by 10 min with propidium iodide staining (1 μg/mL in phosphate buffered saline (PBS)) and analyzed using a fluorescence microscope (Nikon Eclipse TE200, Japan). Apoptotic cells were identified by classical morphological features including nuclear condensation, cell shrinkage, and formation of apoptotic bodies.[36]

Caspase-3 activation was measured using an EnzoLyte AMC Caspase-3 Assay Fluorimetric kit (AnaSpec, Fremont, CA, U.S.A.) according to the manufacturer's instructions.[37] A total of 0.1×10^5 cells were plated in triplicate in a flat-bottomed 96-well plate. Cells were treated with different curcumin-related compounds containing benzyl piperidone for 72 h. Following treatment, caspase-3 substrate was added to each well. Plates were incubated at room temperature for 30 min. Fluorescence intensity was measured in a Tecan Inifinite M200 plate reader (Tecan US Inc., Durham, NC, U.S.A.).

NF-κB-Dependent Reporter Gene Expression Assay An NF-κB luciferase construct (#CLS-013 L, SABiosciences, CA, U.S.A.) was stably transfected into VCaP/N cells and a single stable clone, VCaP/N was obtained and used in the present study. In brief, VCaP/N cells were treated with different curcumin analogues for 24 h, and the NF-κB-luciferase activities were measured using the luciferase assay kits from Promega (Madison, WI, U.S.A.) as described previously.

Western Blot Analysis After treatment with curcumin, PFBr4 for 24 h, PC-3 cells were washed with ice-cold PBS and lysed with 800 μL of lysis buffer (10 mM Tris–HCl, pH 8.0, 10 mM ethylenediamine tetraacetic acid (EDTA), 150 mM sodium chloride, 1% NP-40, 0.5% sodium dodecyl sulfate (SDS), in deionized water). The homogenates were centrifuged at 12000×g for 15 min at 4°C. The protein concentration of whole cell lysates was determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, U.S.A.). Equal amounts (50 μg) of protein were then resolved on a 10% Criterion Precast Gel (Bio-Rad, Hercules) and transferred to a polyvinylidene difluoride (PVDF) membrane using a semi-dry transfer system. The membrane was then probed with with anti-phosphorylated Erk1/2 (#4376, Cell Signaling Technology, U.S.A.) primary antibody. After hybridization with primary antibody the membrane was washed with Tris-buffered saline

three times, then incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and washed with Tris-buffered saline three times. Final detection was performed with enhanced chemiluminescent reagents. The extent of protein loading was determined by blotting for β-actin. The membrane was incubated in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris–HCl at pH 6.7) at 50°C for 30 min with occasional agitation before incubating in blocking buffer and re-probing using anti-β-actin (Santa Cruz Biotechnology).

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