Effects of Curcumin Analogues for Inhibiting Human Prostate Cancer Cells and the Growth of Human PC-3 Prostate Xenografts in Immunodeficient Mice

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Regular Article


Prostate cancer is one of the leading causes of death among men in the United States.10 Prostate carcinogenesis has been viewed as a multistage and complex process consisting of initiation, promotion, and progression. Despite the tremendous efforts and resources devoted to treatment, incidence and mortality rate are still high. Although patients with metastatic prostate cancer can benefit from androgen ablation, most of them will die of prostate cancer progression due to an androgen-refractory state. Hence, much attention has been paid to the discovery of chemopreventive substances from various edible and medicinal plants, a large number of which have been identified.

Curcumin is a non-nutritive yellow pigment found in the spice turmeric, which is derived from the rhizome of the plant Curcuma longa Linn. Numerous studies have demonstrated the anticancer activity of curcumin and curcumin analogues in animal models2–7 as well as growth inhibition and apoptosis induction in a variety of cancer cell lines in vitro.8–16 However, the clinical efficacy of curcumin is limited, which is likely due to its low bioavailability.17–19

We have previously reported on the synthesis and evaluation of sixty-one curcumin-related compounds for inhibitory effects on cultured prostate cancer cells, pancreatic cancer cells and colon cancer cells.20 Four of these curcumin analogues with different linker groups were investigated for their effects in human prostate cancer CWR-22Rv1 and PC-3 cells. Compounds FS and ES had stronger inhibitory effects than curcumin, AS and BS on the growth of cultured CWR-22Rv1 and PC-3 cells, as well as on the androgen receptor (AR) and nuclear factor kappa B (NF-κB) activity. The strong activities of ES and FS may be correlated with a heteroatom linker. In animal studies, severe combined immunodeficient (SCID) mice were injected subcutaneously (s.c.) with PC-3 cells in Matrigel. After 4 to 6 weeks, mice with PC-3 tumors (about 0.6 cm wide and 0.6 cm long) received daily intraperitoneal (i.p.) injections of vehicle, ES and FS (10µg/g body weight) for 31 d. FS had a potent effect in inhibiting the growth and progression of PC-3 tumors. Our results indicate that FS may be useful for inhibiting human prostate tumors growth.

Key words curcumin analog; prostate cancer cell; prostate tumor

Four curcumin analogues ((2E,6E)-2,6-bis(thiophen-3-methylene) cyclohexanone (AS), (2E,5E)-2,5-bis(thiophen-3-methylene) cyclopentanone (BS), (3E,5E)-3,5-bis(thiophen-3-methylene)-tetrahydrothiopyran-4-one (ES) and (3E,5E)-3,5-bis(thiophen-3-methylene)-tetrahydropyran-4-one (FS) as shown in Fig. 1) with different linker groups were synthesized for their effects in human prostate cancer CWR-22Rv1 and PC-3 cells. We found that compounds ES and FS had a stronger inhibitory effect than compound AS and BS on the growth of human prostate cancer CWR-22Rv1 androgen receptor (AR) and PC-3 cells. We also demonstrated that ES and FS inhibited AR and nuclear factor kappa B (NF-κB) activity more potently than curcumin, AS and BS. In animal experiments, we found that FS significantly inhibited the growth of PC-3 tumors.

MATERIALS AND METHODS

Chemistry Four curcumin analogues (AS, BS, ES and FS as shown in Fig. 1) with different linker groups were synthesized by coupling the appropriate substituted benzaldehyde with cyclohexanone, cyclopentanone, tetrahydrothiopyran-4-ones or tetrahydropyran-4-one as previously described.20 Characterization of the compounds, AS, BS, ES, FS, was previously described in detail.20 Curcumin was isolated from the extract of Curcuma longa Linn according to a previous report.21

Cell Culture and Reagents CWR-22Rv1 and PC-3 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.). CWR-22Rv1 cells were maintained in RPMI-1640 culture medium. RPMI-1640 medium was 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% CO2 and were passaged twice a week. Curcumin analogues were dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO was 0.1% in all experiments.

3-(4,5)-Dimethylthiazol-(2-yl)-3,5-diphenyltetrazolium
Bromide (MTT), Trypan Blue and Apoptosis Assays

For the MTT assay, CWR-22Rv1 and PC-3 cells were seeded at a density of $2 \times 10^4$ cells/mL of medium in a 96-well plate (0.2 mL/well) and incubated for 24 h. The cells were then treated with various concentrations (0.5–30 $\mu$M) of curcumin and its analogues 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. For the trypan blue exclusion assay, CWR-22Rv1 and PC-3 cells were seeded at a density of $2 \times 10^4$ cells/mL of medium in 35-mm tissue culture dishes and incubated for 24 h. The cells were then treated with curcumin analogues for 96 h. The number of viable cells after each treatment was determined using a hemocytometer under a light microscope (Nikon Optiphot, Japan). Cell viability was determined by the trypan blue exclusion assay, which was done by mixing 80 $\mu$L of cell suspension and 20 $\mu$L 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.

AR Luciferase Reporter Assay

AR transcriptional activity was measured by an AR-luciferase reporter gene expression assay. An AR luciferase construct was stably transfected into CWR-22Rv1 cells and a single stable clone, CWR-22Rv1/AR, was used in the present study. CWR-22Rv1 cells cultured in 10% FBS RPMI1640 medium were infected with lentivirus carrying Cignal Lenti AR reporter (luciferase) (Qiagen) in the medium containing 8 $\mu$g/mL Polybrene (Sigma). At 6 h after infection, the culture medium was replaced with fresh 10% RPMI1640 medium. To establish the cells expressing stable antioxidative response element (ARE)-luciferase reporter, cells were selected using puromycin (5 $\mu$g/mL) on day 3 after infection for one week. The selected cells were then used for the reporter assay for androgen receptor activity.

CWR-22Rv1/AR cells were treated with curcumin and its analogues for 24 h, and the luciferase activities were measured using luciferase assay kits from Promega (Madison, WI, U.S.A.). After treatment, the cells were washed with ice-cold phosphate buffered saline (PBS) and harvested in a reporter lysis buffer. After centrifugation, 10 $\mu$L aliquots of the supernatants were used for measuring luciferase activity with a luminometer from Turner Designs Instruments (Sunnyvale, CA, U.S.A.). The luciferase activity was normalized against protein concentration and expressed as percent of luciferase activity in the control cells, which were treated with DMSO solvent. The protein level was determined by Bio-Rad protein assay kits (Bio-Rad, Hercules, CA, U.S.A.) according to the manufacturer’s instructions.

NF-κB-Dependent Reporter Gene Expression Assay

An NF-κB luciferase construct (#CLS-013 L, SABiosciences, CA, U.S.A.) was stably transfected into PC-3/N cells and a single stable clone, PC-3/N was obtained and used in the present study. In brief, PC-3/N cells were treated with curcumin and its 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.

Animal Experiment

Male severe combined immunodeficient (SCID) mice were obtained from Taconic Farms, Inc. The animals were housed in sterile filter-capped microisolator cages and provided with sterilized 5010 rodent diet and water. As illustrated in Fig. 2, PC-3 cells ($2 \times 10^6$/0.1 mL per mouse) suspended in 50% Matrigel (Collaborative Research) in RPMI 1640 were injected subcutaneously (s.c.) into the right flank of the mice. After 4 to 6 weeks, mice with PC-3 tumors (about 0.6 cm wide and 0.6 cm long) received daily intraperitoneal
(i.p.) injections of vehicle, ES and FS (10 µg/g body weight) for 31 d. In all experiments, animals in the different experimental groups (5 mice/group) received the same amount of vehicle (5 µL/g body weight), which consisted of a 40:0.5:10:48.5 mixture of propylene glycol, polysorbate 80, benzyl alcohol, ethanol and water. Tumor size (length × width; in square centimeters) and body weight were measured once every 3rd day. All animal experiments were carried out under an Institutional Animal Care and Use Committee-approved protocol. The ANOVA method with the Tukey–Kramer test was used for the comparison of effects among the different treatment groups at the end of the study.

RESULTS

Effects of Curcumin and Its Analogues of CWR-22Rv1 and PC-3 Cells

The inhibitory effects of curcumin analogues on the growth of cultured CWR-22Rv1 and PC-3 cells were determined by using the MTT and trypan blue exclusion assays. For each incubation, curcumin was evaluated as a positive control. Inhibitory effects of different concentrations of curcumin and its analogues in cultured CWR-22Rv1 and PC-3 cells are presented in Fig. 3. All compounds (except BS) had stronger inhibitory effects than curcumin as determined by the MTT assay. Among the four curcumin analogues tested in our study, compounds ES and FS exhibited the most potent inhibitory effects on the growth of cultured CWR-22Rv1 and PC-3 cells. As shown in Table 1, the IC₅₀ values of the four curcumin analogues ranged from 2.4 µM to 23.2 µM. The numbers of viable and dead cells were determined by the trypan blue exclusion assay after treating CWR-22Rv1 and PC-3 cells with curcumin and its analogues (2 µM) for 96 h. As shown

Fig. 3. A. Inhibitory Effects of Curcumin Analogues on the Growth of CWR-22Rv1 Cell Lines; B. Inhibitory Effects of Curcumin Analogues on the Growth of PC-3 Cell Lines

Prostate cancer CWR-22Rv1 and PC-3 cells were seeded at a density of 2×10⁴ 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 (0.5–30 µM) of the different compounds for 72 h. Effects of the different compounds on the growth of CWR-22Rv1 and PC-3 cells were determined by the MTT assay. Each value is the mean±S.E. from three separate experiments.
in Table 2, decreases in the number of viable cells were observed. Compared to the control group, the numbers of viable cells in the various groups were decreased by 52.2% to 88.5% (Table 2).

**Effects of Curcumin and Its Analogues on AR Activity in CWR-22Rv1/AR Cells** We used an AR-luciferase reporter gene expression assay in CWR-22Rv1/AR cells to determine the effect of curcumin and its analogues on the testosterone (TT)-induced activation of AR. Cultured CWR-22Rv1/AR cells were treated with TT in combination with curcumin analogues (1 µM) for 24h. As shown in Fig. 4A, a slight inhibitory effect on the TT-induced increase in AR activity was observed in cultured CWR-22Rv1/AR cells treated with curcumin (1 µM), AS (1 µM) or BS (1 µM), while more obvious inhibitory effects were observed in CWR-22Rv1/AR cells treated with ES (1 µM) or FS (1 µM).

**Table 1. Inhibitory Effects of Curcumin and Its Analogues on the Growth of CWR-22Rv1 and PC-3 Cells**

<table>
<thead>
<tr>
<th>Compound</th>
<th>CWR-22Rv1</th>
<th>PC-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin</td>
<td>17.0±3.0</td>
<td>19.9±2.2</td>
</tr>
<tr>
<td>AS</td>
<td>18.4±2.1</td>
<td>14.6±2.5</td>
</tr>
<tr>
<td>BS</td>
<td>23.2±2.5</td>
<td>23.0±6.0</td>
</tr>
<tr>
<td>ES</td>
<td>3.7±0.5</td>
<td>6.6±1.4</td>
</tr>
<tr>
<td>FS</td>
<td>2.4±0.4</td>
<td>4.1±1.0</td>
</tr>
</tbody>
</table>

Prostate cancer CWR-22Rv1 and PC-3 cells were seeded at a density of 2×10^4 cells/mL in 96-well plates (0.2 mL/well) and incubated for 24h. The cells were then treated with various concentrations (0.5–30 µM) of the different compounds for 72h. Effects of the different compounds on the growth of CWR-22Rv1 and PC-3 cells were determined by the MTT assay. Each value is the mean±S.E. from three separate experiments.

**Table 2. Effects of Curcumin and Its Analogues on the Growth of CWR-22Rv1 and PC-3 Cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of viable cells (1×10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CWR-22Rv1</td>
</tr>
<tr>
<td>Control</td>
<td>36.4±3.9</td>
</tr>
<tr>
<td>Curcumin</td>
<td>21.3±2.5</td>
</tr>
<tr>
<td>AS</td>
<td>23.1±2.4</td>
</tr>
<tr>
<td>BS</td>
<td>23.8±2.6</td>
</tr>
<tr>
<td>ES</td>
<td>17.9±1.8</td>
</tr>
<tr>
<td>FS</td>
<td>15.2±1.7</td>
</tr>
</tbody>
</table>

Prostate cancer CWR-22Rv1 and PC-3 cells were seeded at a density of 2×10^4 cells/mL in 35-mm tissue culture dishes and incubated for 24h. The cells were then treated with curcumin and its analogues (2 µM) for 96h. The number of viable cells was determined by trypan blue exclusion assay.

**Fig. 4. A. Effect of Curcumin Analogues on Testosterone (TT)-Induced Increase in Androgen Receptor Reporter Activity in CWR-22Rv1/AR Cells**

CWR-22Rv1/AR cells were seeded at a density of 1×10^5 cells/mL of medium for 24h. Then the medium was changed to RPMI without FBS, and the cells were treated with vehicle (control) or with testosterone (TT, 100 nM) alone or in combination with curcumin and curcumin analogues (1 µM) for 24h. Luciferase activity and protein concentration of the CWR-22Rv1/AR cells were measured. Each value is the mean±S.E. from three separate experiments.

**B. Inhibitory Effects of Curcumin Analogues on NF-κB Transcriptional Activity in PC-3/N Cells**

PC-3/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 24h. The cells were then treated with 1 µM of the different compounds for 24h. The NF-κB transcriptional activity was determined by the luciferase reporter assay. Each value represents the mean±S.E. from three separate experiments.

**Fig. 5. Effect of i.p. Injections of ES and FS on the Growth of PC-3 Xenograft Prostate Tumors**

Male SCID mice were injected s.c. with PC-3 cells in 50% Matrigel (2.0×10^6 cells/0.1 mL). After 4 to 6 weeks, mice with PC-3 tumors (0.6–1.0 cm wide and 0.6–1.0 cm long) were injected i.p. with ES and FS (10 µg/g body weight/d) for 31d. Tumor size (length×width) and body weight were measured once every 3d and expressed as percent of initial tumor size and percent of initial body weight, respectively. A: growth curve of PC-3 tumors in each group; B: individual body weight of mice after treatment for 31d.
treated with ES (1 μM) and FS (1 μM).

**Effects of Curcumin Analogues on Activation of NF-κB in PC-3/N Cells** To further determine the mechanisms for growth inhibition and apoptosis induction in PC-3 cells, we used an NF-κB-luciferase reporter gene expression assay to determine the effect of curcumin and its analogues on activation of NF-κB. In these experiments, PC-3/N cells were treated with a low concentration (1 μM) of curcumin and its analogues for 24 h. Treatment of PC-3/N cells with group FS compounds resulted in strong decreases in NF-κB transcriptional activity (Fig. 4B). ES had moderate effects for decreasing NF-κB transcriptional activity while curcumin, AS and BS had no effect.

**Effects of i.p. Injections of ES and FS on the Growth of PC-3 Tumors in SCID Mice** Male SCID mice (7–8 weeks old) were injected s.c. with PC-3 cells suspended in a 1:1 mixture of Matrigel and culture medium (2.0×10^6 cells/0.1 mL). When the tumors reached a moderate size (0.6–1.0 cm wide and 0.6–1.0 cm long), the mice received daily i.p. injections of vehicle, ES and FS (10 μg/g body weight/d) for 31 d. The effects of the various treatments on tumor size are described in Fig. 5A. The average tumor size in each group was similar when the experiment started. Statistical analysis using ANOVA with the Tukey–Kramer multiple comparison test showed that the percentage of initial tumor size after 31 d of treatment in the FS-treated group was significantly lower than the control and the ES-treated group (p<0.01). The mean ± S.E. for the percentage of initial tumor size after 31 d of treatment mice was 2.07±0.08 for the control group, 1.94±0.1 for the ES (10 μg/g/d) group, 1.67±0.07 for the FS (10 μg/g/d) group. The results indicate that treatment of the mice with FS had a stronger effect in inhibiting the growth of prostate tumors than treatment with ES and vehicle. The effects of the various treatments on body weight are described in Fig. 5B. The mean (± S.E.) percent of initial body weight after 31 d of treatment was 102.2±1.3% for the control group, 98.1±1.1% for the ES group, 91.2±2.5% for the FS group. Statistical analysis with the Tukey–Kramer multiple comparison test showed that differences in the percent of initial body weight between any two groups were not statistically significant (p>0.05).

**DISCUSSION**

In the present study, we found that curcumin analogues ES and FS had stronger inhibitory effect on the growth of CWR-22Rv1 and PC-3 cells than curcumin, AS and BS. In animal studies, treatment of the mice with ES suppressed the growth of PC-3 tumors. FS had a potent effect on inhibiting the growth of PC-3 tumors. Our results indicate that FS may be a useful compound for inhibiting human prostate tumors growth.

The inhibitory effect of curcumin on carcinogenesis was first shown in a 12-O-tetradecanoylphorbol-13-acetate (TPA)-, BP-, and 7,12-dimethylbenz[a] anthracene-induced mouse skin tumor model. Since then, inhibitory effects of dietary curcumin on prostate cancer, as well as colon, duodenal, stomach, esophageal, and oral carcinogenesis were also reported. Although the opinion about in vivo chemopreventive effects by curcumin is generally held, there exist some reports that contradict this view. For example, Imai et al. have shown that curcumin failed to prevent the formation of ventral prostate carcinogenesis in rats, induced by 3,2-V-dimethyl-4-amino-phenol and 2-amino-1-methylimidazo[4,5-b]pyridine. Somasundaram et al. also have shown that administration of dietary curcumin did not augment cyclophosphamide-mediated growth inhibition of BT-474 breast cancer xenografts but rather contributed to an increase tumor size. Together, these facts suggest that the inhibitory effects of curcumin might vary depending on the animal tumor models, in vivo oral absorption, and/or target organs and that chemopreventive efficacy of curcumin in vivo thus needs to be further evaluated.

Earlier studies using prostate cancer cells in culture showed that ES and FS have significant chemopreventive and possibly chemotherapeutic effects. However, their effects in vivo are still lacking. In the present study, we have shown that i.p. injection of ES and FS significantly retarded the growth of the xenograft tumors, implying that ES and FS possess inhibitory effects on the growth of prostate cancer in vivo. The compounds ES and FS warrant further studies for their potential use in treatment of prostate cancers.

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