Synthesis and biological analysis of new curcumin analogues bearing an enhanced potential for the medicinal treatment of cancer

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Abstract
Curcumin (diferuloylmethane) is a dietary phytochemical with low toxicity that exhibits growth-suppressive activity against a variety of cancer cells and possesses certain chemopreventive properties. Curcumin has already been the subject of several clinical trials for use as a treatment in human cancers. Synthetic chemical modifications of curcumin have been studied intensively in an attempt to find a molecule with similar but enhanced properties of curcumin. In this study, a series of novel curcumin analogues were synthesized and screened for anticancer activity. New analogues that exhibit growth-suppressive activity 30 times that of curcumin and other commonly used anticancer drugs were identified. Structurally, the new analogues are symmetrical 1,5-diarylpentadienone whose aromatic rings possess an alkoxy substitution at each of the positions 3 and 5. Analysis of the effects of the analogues on the expression of cancer-related genes usually affected by curcumin indicated that some induced the down-regulation of β-catenin, Ki-ras, cyclin D1, c-Myc, and ErbB-2 at as low as one eighth the concentration at which curcumin normally has an effect. The analogues, however, exhibited neither harmful nor growth-suppressive effects on normal hepatocytes where oncogene products are not activated. They also exhibited no toxicities in vivo that they may provide effective alternative therapies for the prevention and treatment of some human cancers. [Mol Cancer Ther 2006;5(10):2563–71]

Introduction
Many anticancer therapies currently in use are inadequate not only in terms of their therapeutic efficacy but also because they have undesirable side effects. On the other hand, certain dietary constituents known as phytochemicals have been shown to exhibit growth-suppressive activity and chemopreventive properties against various types of cancers (1) without the adverse side effects normally associated with current chemotherapies. Curcumin is one of the most widely characterized of the phytochemicals, exhibiting both growth-suppressive potential in a wide variety of tumor cells and a chemopreventive effect in certain types of cancer, such as colon and skin cancers (2, 3).

The mechanism of action of curcumin has been extensively studied at the molecular level (4). It is known that curcumin interferes with the transcriptional activation induced by transcription factors, such as nuclear factor-κB (NF-κB; ref. 5) and activator protein-1 (6), resulting in the negative regulation of various cell cycle control genes and oncoproteins, such as c-Myc, cyclin D1, Bcl-2, and Bcl-XL (4). Cyclooxygenase-2, which is overexpressed in colorectal cancers through NF-κB or activator protein-1 transactivation, is also suppressed by curcumin (7), and other cyclooxygenase-2 inhibitors are known to have chemopreventive and antiangiogenic properties (4). Curcumin has been shown to arrest the cell cycle at G0-G1 or G2-M through up-regulation of the cyclin-dependent kinase inhibitors p21 and p27 and down-regulation of Cdc2 and cyclin B1 (8). Curcumin blocks growth factor signaling via inhibition of tyrosine kinase activity or depletion of ErbB-2 (9). More recently, it has been shown that curcumin causes the cleavage of β-catenin, resulting in apoptosis in a colon cancer–derived cell line (10, 11). Loss of function of the APC tumor suppressor gene, which is mutated in most colon cancers, inhibits β-catenin degradation (12) and the resulting accumulation of β-catenin in the cytosol that is translocated to the nucleus causing transactivation of oncoproteins, including c-Myc and cyclin D1 (13). The loss of the APC gene function and subsequent accumulation of β-catenin is therefore believed to be the initiation event of colorectal carcinogenesis (14, 15). Hence, the ability of curcumin to target β-catenin for degradation is considered to be the basis of the chemopreventive effect of curcumin in colorectal cancer. It was found that curcumin treatment...
reduced the incidence of adenoma formation in the familial adenomatous polyposis mouse model to 40% of control (2).

However, in clinical trials of oral administration of curcumin to human cancer patients, the systemic availability of curcumin was found to be negligible, especially outside the gut, due to poor absorption of the compound (16, 17). We synthesized and tested the growth-suppressive ability of >50 synthetic analogues of curcumin to increase the potentials of curcumin and circumvent the low bioavailability while keeping its low toxicity. Several derivatives showed an enhanced ability to induce apoptosis in different cancer cell lines. These derivatives also decreased the expression levels of oncoproteins, including β-catenin, Ki-ras, cyclin D1, and ErbB-2, at concentrations much lower than those normally used for curcumin.

Materials and Methods

Compounds

Chemical synthesis, physical properties, and molecular formulas of the new derivative compounds are published as supporting information (Supplementary Fig. S1). Curcumin (Sigma-Aldrich, Inc., St. Louis, MO) and its analogues were dissolved in DMSO at 50 mmol/L as stock solution. Caspase-3/caspase-8 inhibitor N-CBZ-ASP-GLU-VAL-ASP fluoromethyl ketone (Z-DEVD-fmk) was purchased from Sigma-Aldrich.

Cell Lines and Culture Conditions

All cell lines, except below, were obtained from the Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan). HCT116 carrying wild-type p53 [HCT116 cells (p53⁺/⁺)] and HCT116 lacking wild-type p53 [HCT116 cells (p53⁻/⁻)] were a kind gift from Dr. B. Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD; ref. 18). Normal human primary hepatocytes (hNHePs) were purchased from Cambrex Bio Science Walkersville, Inc. (East Rutherford, NJ).

Cell Growth Suppression Analysis

Growth-suppressive effects of the derivative compounds were measured in different cancer cell lines for 96 hours. Cell viability was assayed by quantitation of the uptake and digestion of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfolphenyl)-2H-tetrazolium monosodium salt according to the manufacturer’s instructions (Dojindo Laboratories, Kumamoto, Japan) by 96-well plate reader, MPR-A4i (Tosoh Corp., Tokyo, Japan). The percentage cell growth of the control, which was treated with 1% DMSO alone, was calculated and plotted, and then the mean growth-inhibitory concentration (IC₅₀) value was determined.

Cell Cycle Analysis

Cell cycle phase was determined by fluorescence-activated cell sorting analysis. Cells from the cell line HCT116 were inoculated into six-well plates at a concentration of 5 × 10⁴ per well, exposed to the derivative compounds at their various concentrations, cultured for 30 hours, collected, and sorted using a FACSscan flow cytometer (Beckman Coulter, Inc., Fullerton, CA) as described previously (19). The percentage of each cell fraction corresponding to the sub-G₁, G₀-G₁, S, and G₂-M phases was calculated using MacCycler (Phoenix Flow Systems, San Diego, CA).

Caspase-3-Like Activity

The induction of caspase-3-like activity was measured by fluorescence as described previously (20). In brief, cells were treated with curcumin analogues for 24 hours, washed with PBS, lysed in a buffer containing 0.5% NP40, 10 mmol/L HEPES (pH 7.4), 2 mmol/L EDTA, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 5 μg/mL leupeptin, and then spun. The caspase-3 substrate N-acetyl-ASP-GLU-VAL-ASP-7-amido-4-methylcoumarin (50 μmol/L; Calbiochem, La Jolla, CA) was incubated with the supernatant containing 250 μg of total protein at 37°C for 30 minutes. Fluorescence was measured at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Caspase-3-like activity was represented as fluorescence units per minute per milligram of protein.

NF-κB Transactivation

NF-κB transactivation was measured by ELISA using a NF-κB p50 kit (Stressgen Bioreagents, Victoria, British Columbia, Canada) according to the manufacturer’s instructions. Shortly, cells were treated with curcumin analogues for 8 hours, washed with PBS, and lysed in the buffer containing radioimmunoprecipitation assay extraction reagents. Whole-cell extracts containing 25 μg protein were applied to the assay kit. The chemiluminescence derived from the active form of NF-κB p50 was measured using a CCD camera (Las-1000, Fuji Photo Film Co. Ltd., Tokyo, Japan).

Expression Profile

Total RNA was extracted from HCT116 treated with compounds using an RNeasy mini kit (Qiagen, Inc., Chatsworth, CA). Total RNA (500 ng) was amplified and labeled using low RNA input linear amplification and a labeling kit according to the manufacturer’s protocol (Agilent Technologies, Inc., Palo Alto, CA). For cRNA labeling, cyanin 3-CTP (Cy3) and cyanin 5-CTP (Cy5; Perkin-Elmer, Inc., Wellesley, MA) were used. Except for the dye swap experiment, controls were labeled with Cy3 and samples were labeled with Cy5. The integrity of the labeled cRNA and labeling index of the dye were confirmed by a NanoDrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). Labeled cRNA (1 μg) was hybridized with the microarray with 41,058 human cDNA oligonucleotides (Human Whole Genome Oligo Microarray kit with SuperPrint Technology, Agilent Technologies) according to the manufacturer’s protocol. After washing, the array slide was scanned using ScanArray 5000 (GSI Group, Inc., Wilmington, MA). Images were processed and signals were quantitated using ArrayVision version 8 (Amersham Biosciences Corp., Piscataway, NJ). The obtained data were normalized using...
Lowess algorithm of GeneSpring version 7 (Agilent Technologies). Spots with a signal/background ratio <2.0 in at least five of seven experiments were excluded from the data analysis. Finally, 16,555 genes were selected and analyzed. The expression level of each gene was represented as the relative value to the control.

**Western Blotting**

Antibodies used for Western blotting (19) were anti-actin monoclonal antibody (A2066, Sigma-Aldrich), anti-β-catenin monoclonal antibody (610153, BD Biosciences, San Jose, CA), anti-cyclin D1 (M20), c-Myc (9E10), Ki-ras [K-Ras-2B (C-19)], and p53 (FL393) monoclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and an anti-c-ErbB-2 monoclonal antibody (A0485, DakoCytomation Denmark A/S, Glostrup, Denmark).

**Animal Experiments**

Inbred mouse strain, C57BL/6J, was obtained from CLEA Japan, Inc. (Tokyo, Japan) and fed with 5 g/d high fat diet (HFD32, CLEA Japan) mixed with 0.1% GO-Y030 or GO-Y031 (w/w). Animal experiments were done following approval from institutional guideline.

**Results**

**Screening of Phytochemical Analogues**

Dietary phytochemicals, including curcumin, resveratrol, capsaicin, caffeic acid phenethyl ester, [6]-gingerol, diallyl sulfide, epigallocatechin-3-gallate, and indole-3-carbinol, are known to have both growth-suppressive and chemopreventive activity against specific types of cancers (1). From our synthetic organic compound library composed of >2,000 species, we selected 45 compounds structurally analogous to the phytochemicals mentioned above and tested their abilities to suppress the growth of the colon cancer cell line DLD-1. Only one compound, GO-035, which is nominated as an analogue of curcumin, was found to have a stronger ability to suppress the growth of DLD-1 compared with curcumin (Figs. 1A and 2A). The IC\(_{50}\) value of GO-035 was 2.0 μmol/L, a value four times lower than that of curcumin (IC\(_{50}\), 8.0 μmol/L; Fig. 2A). The growth-suppressive activity of GO-035 in two other colon cancer cell lines, SW620 and HCT116 cells (p53\(^{+/−}\)), was also examined (Fig. 2B and C). In these cases, both the IC\(_{50}\) values of GO-035 were again lower than that of curcumin. The IC\(_{50}\) values of GO-035 in SW620 and HCT116 cells (p53\(^{+/−}\)) were 3.5 and 1.6 μmol/L, respectively, and those of curcumin were 10.0 and 6.5 μmol/L, respectively. The growth-suppressive activity of GO-035 was examined in other cancer cell lines, including lines derived from stomach (GCIY, SH10TC), lung (LK87), breast (MCF7), ovary (OVK18), prostate (PC3), pancreas (PK9), bile duct (HuCCT1), thyroid gland (8505c), skin (A431), kidney (ACHN), and liver cancers (HepG2) and also melanoma (G361; Fig. 3A). Curcumin exhibited growth-suppressive activity in HCT116 cells (p53\(^{+/−}\)), SW620, GCIY, LK87, MCF7, PK9, 8505c, and G361 than curcumin in the same cell lines at the concentrations tested (Fig. 3A).

**Growth-Suppressive Potential of New Curcumin Analogues**

A panel of related compounds was also synthesized and examined. Four series of curcumin analogues were designed and synthesized aiming not only to identify crucial structural motifs leading to growth-suppressive ability for carcinogenesis but also to gain insight into directions for designing new derivatives with increased activities: (a) curcumin-type compounds that retain the 7-carbon spacer between the aryl rings (diarylheptanoids), (b) GO-035-type compounds that have a 5-carbon spacer between the aryl rings (diarylpentanoids), (c) GO-035-type compounds in which conformational freedom is fixed by the central cyclic ketone structure, and (d) others (Fig. 1B; Supplementary Fig. S2; Supplementary Table S1). 5 Results
during the course of this study suggested that (a) the methyl modification of the $p$-hydroxy group relative to the $\alpha,\beta$-unsaturated ketone moiety leads to considerable enhancement in the growth-suppressive activity (e.g., GO-Y025 > curcumin), (b) a 5-carbon tether is superior to a 7-carbon tether (e.g., GO-035 > GO-025), and (c) conformational fixation around the enone subunit leads to significant attenuation of the activity (e.g., GO-035 > GO-032; Fig. 1A and B). Hence, derivatives in series 2 were synthesized to identify new compounds with enhanced activity. Fifty-one compounds, including 33 diarylpentanoids, were newly synthesized and their growth-suppressive effect on HCT116 cells (p53+/+) was examined (Fig. 1B; Supplementary Table S1). The IC$_{50}$ values of series 2 compounds ranged from 0.25 to over 100 $\mu$mol/L (Fig. 1B; Supplementary Table S1). Among them, 34 compounds showed a higher potential to suppress growth in HCT116 cells (p53+/+) compared with curcumin, and 18 of the 34 compounds showed higher growth-suppressive activity compared with GO-035. These compounds had 1,5-diarylpentadienone skeleton as the common structural motif, and it was indicated that the introduction of suitable alkoxy groups on the aromatic rings led to an increase in the growth-suppressive potential. Among the diarylpentanoids, compounds GO-Y016, GO-Y030, and GO-Y031 showed the highest growth-suppressive activity in HCT116 cells (p53+/+). The IC$_{50}$ value of these three compounds was 0.25 $\mu$mol/L (Fig. 2; Supplementary Table S1). This low value corresponded to 1/32 of the IC$_{50}$ value of curcumin and 1/8 that of GO-035. The IC$_{50}$ values of GO-Y016 in all the cell lines tested ranged from 0.10 to 0.50 $\mu$mol/L, except A431 and HepG2, where the IC$_{50}$ value was 2.0 $\mu$mol/L in each case (Fig. 3A). GO-Y016 exhibited 12 to 60 times higher growth-suppressive activity than the highest activities of curcumin. GO-Y016 exhibited 4.8 to 18.0 times higher growth-suppressive activity compared with GO-035. Similar growth suppression activities were observed for both GO-Y030 and GO-Y031 (Fig. 3A). GO-Y030 exhibited 8.0 to 40.0 times and GO-Y031 exhibited 13.2 to 50.0 times higher growth-suppressive activities compared with curcumin in the cell lines where growth suppression occurred. Compared with GO-035, GO-Y030 exhibited 3.0 to 13.5 times and GO-Y031 exhibited 3.0 to 23.3 times the growth-suppressive activity (Fig. 3A). Each of the compounds GO-Y016, GO-Y030, and GO-Y031 had fundamentally stronger growth suppression activities in cancer cell lines than curcumin or GO-035.

**Growth-Suppressive Activities of New Curcumin Analogues Compared with Currently Used Anticancer Drugs**

We compared the growth-suppressive activities of GO-Y016, GO-Y030, and GO-Y031 with the most commonly used chemotherapeutic agents [i.e., 5-fluorouracil (5-FU), CDDP, and CPT-11]. IC$_{50}$ values of 5-FU on 16 cancer cell lines ranged from 0.35 to 10.0 $\mu$mol/L (Fig. 3B). Comparison of the growth-suppressive potential between 5-FU and these three diarylpentanoids was carried out by calculating the ratio of IC$_{50}$ value of 5-FU to that of diarylpentanoid
[IC₅₀ ratio (5-FU/diarylpentanoid)]. In LK87, PC3, HuCCT1, and A431, there was no apparent difference between the growth-suppressive activities of 5-FU and the diarylpentanoids. The IC₅₀ ratios (5-FU/diarylpentanoid) in the other 12 cell lines ranged from 6.0 to 80.0, except for the IC₅₀ ratio (5-FU/GO-Y016) in cell line HepG2 (2.5 μmol/L). GO-Y016, GO-Y030, and GO-Y031 were able to induce a stronger growth suppression at a much lower concentration than 5-FU in the majority of cancer cell lines. The IC₅₀ values of CDDP on 16 cell lines ranged from 0.16 to 17.0 μmol/L. In cell lines GCIY, LK87, PC3, and PK9, the IC₅₀ values ranged from 0.16 to 0.7 μmol/L. They were relatively sensitive to CDDP. The IC₅₀ ratios (CDDP/diarylpentanoid) in MCF7, HuCCT1, and 8505c ranged from 17.5 to 60.0, indicating that GO-Y016, GO-Y030, and GO-Y031 could induce stronger growth suppression at a concentration lower than that of CDDP in some types of cancer. The IC₅₀ values of CPT-11 on 16 cell lines ranged from 0.5 to 10.0 μmol/L. The IC₅₀ ratio (CPT-11/diaryl pentanoid) of DLD-1, SH10TC, MCF7, and 8505c ranged from 10.0 to 33.3, indicating that GO-Y016, GO-Y030, and GO-Y031 could induce stronger growth suppression at a concentration lower than that of CPT-11 in some types of cancer.

**The Effect of New Curcumin Analogues on Cell Cycle Progression**

The effect of each diarylpentanoids on cell cycle progression was examined by fluorescence-activated cell sorting analysis (Fig. 4). HCT116 cells (p53+/−) were treated with curcumin, GO-035, GO-Y016, GO-Y030, and GO-Y031. The concentrations of compounds were chosen from the growth suppression experiments showing moderate toxicity. As shown in Fig. 4, 30 hours of treatment induced a significant effect in each experiment. As shown previously (10), 52% of the cell population arrested in the G₂-M phase at 20 μmol/L curcumin. Conversely, the cell population at the G₀-G₁ phase was reduced to 7% probably due to the G₂-M arrest, but the S phase fraction did not change. GO-035 treatment at 5 μmol/L had the same effect on cell cycle progression, where 47% of the cell population arrested in the G₂-M phase, and the G₀-G₁ phase fraction was reduced to 7%.

The S phase fraction did not change at 5 μmol/L GO-035 treatment. On the other hand, GO-Y016, GO-Y030, and GO-Y031, which have the highest growth-suppressive activities among the diarylpentanoids, exerted different effects on cell cycle progression. GO-Y016 was difficult to dissolve; thus, only 1 μmol/L could be assessed. For 1 μmol/L GO-Y016, the same degree of G₂-M arrest was observed as seen in 20 μmol/L curcumin. G₂-M arrest was also observed with 2 μmol/L GO-Y030 and GO-Y031 treatment. The most drastic change with GO-Y016 was the reduction of the S phase fraction to 28% of the cell population, whereas the S phase fraction of the control, curcumin, and GO-035 was 46%, 41%, and 46%, respectively. This tendency was more apparent in the cases of 2 μmol/L GO-Y030 and GO-Y031 treatment, where the reduction of the S phase fraction was 15% and 10%, respectively. Furthermore, the sub-G₁ fraction (20% and 26% of the cell population) additionally appeared following GO-Y030 and GO-Y031 treatment, respectively. The reduction of the S phase fraction corresponded to the inhibition of DNA synthesis, whereas the elevation of the sub-G₁ fraction corresponded to the induction of apoptosis. We suggest that the activities of GO-Y030 and GO-Y031 were reinforced in these two aspects.

**Caspase-3-Like Activity with the New Curcumin Analogues**

The induction of caspase-3-like activity with new curcumin analogues was examined. Caspase-3 is one of the major components of the apoptosis pathway, including curcumin-related apoptosis. Caspase-3-like activity was measured by measuring the concentration of 7-amido-4-methylcoumarin generated by cleavage following treatment with curcumin and its new analogues. New curcumin analogues, such as GO-035 (5 μmol/L), GO-Y016 (2.5 μmol/L), GO-Y030 (2.5 μmol/L), and GO-Y031 (2.5 μmol/L), showed 67.38 ± 1.03, 56.93 ± 3.35, 81.45 ± 5.29, and 67.33 ± 3.30 arbitrary fluorescent units/min/mg caspase-3-like activities that are 1.33, 1.12, 1.61, and 1.33 times higher than 20 μmol/L GO-035 caspase-3-like activity, respectively (curcumin, 50.69 arbitrary fluorescent units/min/mg caspase-3-like activity) with 20 μmol/L curcumin treatment, respectively. The most drastic increase was seen in 20 μmol/L GO-Y030 and GO-Y031 treatment, where the reduction of the S phase fraction was 15% and 10%, respectively. Furthermore, the sub-G₁ fraction (20% and 26% of the cell population) additionally appeared following GO-Y030 and GO-Y031 treatment, respectively. The reduction of the S phase fraction corresponded to the inhibition of DNA synthesis, whereas the elevation of the sub-G₁ fraction corresponded to the induction of apoptosis. We suggest that the activities of GO-Y030 and GO-Y031 were reinforced in these two aspects.

Effects of New Curcumin Analogues on Gene Expression

Much is known about the effects of curcumin on gene expression at the transcriptional and posttranscriptional level (4, 9, 10, 21). The suppression of NF-κB transactivation is one of the biological effects by curcumin. The relative level of NF-κB transactivation was 0.24 ± 0.02 at 20 μmol/L curcumin treatment, whereas they were 0.59 ± 0.07 and 0.53 ± 0.07 at 2.5 μmol/L GO-Y030 and GO-Y031 treatment, respectively (Supplementary Fig. S4).5 Suppression of NF-κB transactivation was observed in a dose-dependent manner for curcumin. On the other hand, the extent of suppression was rather weak with GO-Y030 and GO-Y031 even at the concentration where the biological effect was apparent. These results indicate that the suppression of NF-κB transactivation is not directly involved in the enhancement of growth-suppressive activities seen in the new curcumin analogues. Then, the comprehensive expression profiling affected with these compounds was estimated by using microarray analysis. Almost curcumin-related genes described in the literature were spotted within 2-fold variation compared with curcumin when treated with GO-Y030 or GO-Y031 (Supplementary Fig. S5A and B).5 Among them, the expression levels of the target genes of NF-κB transactivation were not always suppressed, rather stable with few exceptions, such as Bcl-2 in GO-Y030 or c-Myc in GO-Y031 cases (Supplementary Fig. S5C).5 The expression level of β-catenin was stable after the treatment of these compounds. On the other hand, the expression levels of several other genes, including ErbB-2 and Ki-ras, were down-regulated when treated with curcumin and its analogues, whereas the level of TP53 was up-regulated in the cases of GO-Y030 and GO-Y031 (Supplementary Fig. SSD).5 To validate the effect on the expression levels of these oncogenes with the new curcumin analogues, Western blot analyses were carried out. ErbB-2 expression completely disappeared with each treatment of 5 μmol/L GO-035, 2.5 μmol/L GO-Y030, and 2.5 μmol/L GO-Y031 as well as 20 μmol/L curcumin (Fig. 5A). c-Myc expression was down-regulated to 50% of control with each treatment of 5 μmol/L GO-035 and 2.5 μmol/L GO-Y030 as well as 20 μmol/L curcumin, except 2.5 μmol/L GO-Y031 treatment where the reduction of c-Myc expression was 80% of control. Cyclin D1 expression was maximally down-regulated by 5 μmol/L GO-035 and 2.5 μmol/L GO-Y030 treatments to 30% of control. GO-Y031 (2.5 μmol/L) and curcumin (20 μmol/L) down-regulated the gene to a lesser extent to 60% and 80% of control, respectively. In the cases of c-Myc and cyclin D1, the transcriptional levels of these genes were stable, but they were down-regulated at the protein level. Curcumin is also well studied to induce the degradation of β-catenin. Similar levels of β-catenin degradation were observed at 5 μmol/L GO-035, 2.5 μmol/L GO-Y030, and GO-Y031 treatment as well as 20 μmol/L curcumin treatment (Fig. 5B). The expression level of Ki-ras was reduced with curcumin as well as its new analogues, those were reduced to 30%, 20%, 20%, and 50% of control in 20 μmol/L curcumin, 5 μmol/L GO-035, 2.5 μmol/L GO-Y030, and GO-Y031 treatments, respectively (Fig. 5B). This is the first evidence that curcumin and its analogues successfully induced the reduction of the activated Ki-ras in the colorectal cancer cell line (22). Previously, it has been

Figure 5. Western blot analyses of the genes affected with the new curcumin analogues. A, protein levels of ErbB-2, c-Myc, and cyclin D1 affected with curcumin and its analogues in HCT116. B, expression of β-catenin and Ki-ras with curcumin and its analogues, HCT116 cells (p53+/+) were treated with the compounds at the indicated concentrations for 30 h with (lanes 6 – 10) or without (lanes 1 – 5) Z-DEVD-fmk pretreatment. Bracket, β-catenin breakdown products. C, stability of p53 treated with the new curcumin analogues. The stability of p53 was examined 6, 12, and 30 h after exposure. Quantification of each expression level was carried out by Image Gauge version 3.0 (Fuji Photo Film) standardized by the value of β-actin (Actin) and represented as a relative value to the control (1% DMSO alone; lane 1). Arrows, protein sizes.
shown that caspase-3 inhibition blocked β-catenin degrada-
tion with curcumin and that caspase-3 plays a crucial role in curcumin-induced β-catenin degradation but nei-
ther proteasomal nor lysosomal pathway (10). To examine the involvement of caspase-3 in β-catenin degrada-
tion as well as Ki-ras, cyclin D1, and c-Myc with the new curcumin analogues, Z-DEVD-fmk was applied. Pretreatment of 20 μmol/L Z-DEVD-fmk completely blocked the β-catenin degradation with GO-035, GO-Y030, and GO-Y031 as well as curcumin (Fig. 5B). The down-regulation of Ki-ras expression with curcumin, GO-035, GO-Y030, and GO-
Y031 was also completely blocked by Z-DEVD-fmk treat-
ment (Fig. 5B). The down-regulation of ErbB-2, c-Myc, and cyclin D1 was not completely blocked by Z-DEVD-fmk treatment (data not shown). The protein level of p53 with curcumin seems to depend on the cell types, which is overexpressed in some cell types and down-regulated in the others (23–25). We examined the effect of the new curcumin analogues on p53 expression level in HCT116 cells (p53+/−) as well as curcumin (Fig. 5C). Curcumin reduced the expression level of p53 to 5% of control during the first 6 hours after exposure, and then the expression level gradually recovered (Fig. 5C). However, for GO-Y030, p53 was relatively stable and its level was reduced to as low as 40% of control during the first 6 hours. For GO-Y031, the result was between that of curcumin and GO-Y030. After the temporal reduction, overexpression of p53 was observed in all cases of curcumin analogues at 30 hours after exposure (Fig. 5C). As shown above, the transcriptional, posttranscriptional, or both mechanisms regulate the gene expression affected by curcumin and its analogues. The regulatory mechanisms varied individually among the genes. To examine the biological significance of the overexpression of p53, we compared the IC50 values of GO-Y030 between HCT116 cells (p53+/−) and HCT116 cells (p53−/−). The IC50 values of HCT116 cells (p53+/−) and HCT116 cells (p53−/−) were 0.18 and 0.23 μmol/L, respectively (Fig. 2A and D). It was considered that there was no relationship between p53 overexpression and the enhanced apoptosis with GO-Y030. The biological significance of p53 overexpression of GO-Y030 remains to be elucidated.

Safety of the New Curcumin Analogues

To evaluate the growth suppression of these new curcumin analogues against the normal cells, the primary human hepatocyte hNHeps was treated with new curcumin analogues. Even at concentrations as high as 100 μmol/L, GO-035 and GO-Y030 showed almost no suppression against primary hepatocytes similar to the effects observed with the most common doses of curcumin (Fig. 6A–C). For GO-Y031, growth suppression was observed to some extent at 100 μmol/L (Fig. 6D). In comparison with cancer cells, such as HCT116, these new curcumin analogues were less growth suppressive and harmless against the normal hepatocytes. Moreover, we examined the toxicity of these compounds in mice when given orally at a dose of 0.1% (w/w) daily, which dose was applied in case of curcumin (2). Judging from the body weight, behavior, and the appearance, there were no adverse effects on either mouse groups fed with GO-030 or GO-Y031 during 45 days (Fig. 6E). The longest exposure reaches at over 120 days without any changes.

Discussion

Curcumin is a dietary phytochemical that is less toxic and has an ideal potential to down-regulate the critical genes activated in cancer. However, it has some short points, including its low bioavailability claimed in vivo. These characters of curcumin are encouraging investigators to modify it into more aggressive forms to induce tumor suppression (23, 26, 27). Our strategy is to develop the new curcumin analogues systemically. As the results of the first screening, one direction of development, in which the 7-carbon tether of curcumin is converted to 5-carbon tether, was chosen. During the course of our work, we noted that Bowen et al. and the Shoji-Snyder team published results on the high degree of anticancer activity of diarylpenta-
noids, including a molecule identical to GO-035 (26, 27). However, our studies indicate that the second direction in which the location and dimensions of the substitutions on Figure 6. Safety of the new curcumin analogues. Growth-suppressive potential of curcumin analogues was evaluated against the primary hepatocyte (hNHeps; □ compared with HCT116 cells (p53+/−); ●, A, curcumin, B, GO-035, C, GO-Y030, D, GO-Y031. E, safety of the new analogues (GO-Y030 and GO-Y031) in vivo was assessed by the body weight.
New Curcumin Analogues Bearing an Enhanced Potential

the aromatic rings are also important. The symmetrical introduction of a pair of alkoxy groups at positions 3 and 5 seems to confer considerable growth-suppressive potentials to the compounds. Several new analogues of curcumin were created in this study, some of which have shown very promising growth-suppressing properties. GO-Y030 and GO-Y031 have a higher capacity for growth suppression in many cancer cell lines than has been reported in curcumin analogues to date. Some points of success of the modification were consistent with previous reports (26, 27). It was also proposed that the unsaturated structures, such as α,β-unsaturated ketone moiety, are likely to act as Michael acceptors (26, 27); however, the effects of these structural modifications on the cellular biological reactions have not been described thus far. This is the first time that an α,β-unsaturated ketone modification has been shown to be important in cell growth regulation. The modification of curcumin to a diarylpentanoid and some types of the substitutions of a pair of alkoxy groups to the phenolic rings were the primary causes of the increase in the growth-suppressive activity of these molecules. GO-Y016, GO-Y030, and GO-Y031 were able to induce stronger growth suppression at a concentration much lower than that of 5-FU in the majority of cancer cell lines. GO-Y016, GO-Y030, and GO-Y031 could induce stronger growth suppression at a concentration lower than those of CPT-11 and CDDP in some types of cancer. Curcumin is a multifunctional compound that affects dozens of molecules, but the precise mechanism to suppress tumors is still unknown (4, 9, 10, 21) and further work is needed to identify the entire molecules that are directly affected with curcumin. We applied expression profile analysis to these new curcumin analogues as the first clue to resolve these issues. Especially, the mechanism of enhancement of the growth-suppressive effects of these new curcumin analogues must be open at the molecular level. GO-035, GO-Y030, and GO-Y031 have a stronger potential to induce down-regulation of oncoproteins, including β-catenin, ErbB-2, c-Myc, cyclin D1, and Ki-ras, than curcumin. Inhibition of caspase-3-like activity diminishes the potentials of curcumin and its analogues to induce apoptosis, the degradation of β-catenin, and the down-regulation of Ki-ras. Therefore, the increased induction of caspase-3-like activity could account for not only the observed increase in apoptosis but also the growth suppression of cancer cells through the down-regulation of oncoproteins, such as β-catenin and Ki-ras. In the latter case, caspase-3 might cleave Ki-ras as well as β-catenin. We concluded that the enhanced caspase-3-like activity plays one of the important roles to enhance the potentials of new analogues. Up to now, in the cases of down-regulation of Ki-ras and β-catenin, it is defined that these genes affected with curcumin and its analogues are regulated posttranscriptionally through caspase-3-dependent pathway. On the other hand, G2-M arrest was similarly observed in the presence and absence of the caspase-3/caspase-8 inhibitor. The down-regulation of ErbB-2, c-Myc, and cyclin D1 is also independent from caspase-3 pathway. Furthermore, we gave but not gained a complete understanding of the underlying mechanisms about apoptosis. For example, it has not been proven that apoptosis and the degradation of β-catenin with new analogues are mediated via the Fas receptor as previously shown with curcumin, which is located upstream of the caspase-3/caspase-8 pathway (28). Moreover, the over-expression of p53 does not contribute to apoptosis because the enhanced apoptosis induction with GO-Y030 was similarly observed in HCT116 cells (p53+/−).

If the other critical target of curcumin could be identified, information about the interaction between the molecular surface of the target and the shape of the compound using computational analysis might be useful for designing further, even more effective, compounds. The abilities of these analogues to reduce or diminish the levels of β-catenin and Ki-ras expression that are particularly involved in the initiation or early steps of carcinogenesis suggest that they may be useful as a means of reducing the incidence of certain cancers, such as colorectal carcinogenesis. It is observed that there is no adverse reaction in vivo with these new compounds. Advancement of the potentials for the growth suppression and chemoprevention of these compounds could result in the improvement of the poor bioavailability of curcumin. In vivo evaluation of these compounds using animal models for several cancers should be done to clarify this point.

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References


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