Table 3. Hematological adverse reactions after the first cycle

Toxicity	Dosage level	No. of patients	Grade of adverse reaction				Incidence of grade 4 (%)
			1	2	3	4	
Leukopenia	1	6	1	1	1	0	0
	2	6	1	2	2	0	0
Neutropenia	1	6	0	1	0	1	16.7
	2	6	1	1	1	2	33.3
Febrile neutropenia	1	6	0	0	0	0	0
	2	6	0	0	0	0	0
Thrombocytopenia	1	6	2	0	0	0	0
	2	6	0	3	1	0	0
Anemia	1	6	0	1	1	0	0
	2	6	0	3	0	0	0

All hematological adverse reactions after the first cycle are listed. Grade 4 was observed only for neutropenia without fever, and others were mild
Grade is according to the NCI-CTC (National Cancer Institute common toxicity criteria)

Table 4. Nonhematological adverse reactions after the first cycle

Toxicity	Dosage level	No. of patients	Grade of adverse reaction				Incidence of grade 3 or 4 (%)
			1	2	3	4	
Anorexia	1	6	0	1	1	0	16.7
	2	6	0	0	0	0	0
Nausea	1	6	0	0	1	0	16.7
	2	6	0	0	0	0	0
Fatigue	1	6	1	0	0	0	0
	2	6	0	0	0	0	0
Creatinine	1	6	0	0	0	0	0
	2	6	1	0	0	0	0
Edema	1	6	0	0	0	0	0
	2	6	1	0	0	0	0
SGOT	1	6	0	0	0	0	0
	2	6	1	0	0	0	0
SGPT	1	6	0	0	0	0	0
	2	6	0	1	0	0	0
Injection site reaction	1	6	0	0	0	0	0
	2	6	1	0	0	0	0
Alopecia	1	6	1	0	0	0	0
	2	6	0	0	0	0	0
Neuropathy (sensory)	1	6	0	0	0	0	0
	2	6	0	0	0	0	0

All nonhematological adverse reactions after the first cycle are listed. Grade 3 adverse reactions were observed for anorexia and nausea, but others were mild
Grade is according to the NCI-CTC (National Cancer Institute common toxicity criteria)
SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase

had relapsed or refractory lesions, and the sites of the lesions were: five esophageal recurrences, eight lymph nodes (one cervical, five mediastinal, and two abdominal), three lung metastases, one liver metastasis and one pleuritis carcinomatosa.

All patients were assessable for toxicity. Hematological and non-hematological adverse events and DLTs are shown in Tables 3, 4, and 5, respectively. The only grade 4 hematological adverse event observed in this study was neutropenia without fever, and other adverse events were mild. Grade 3 non-hematological adverse events were anorexia and nausea. Fatigue, renal and liver toxicities, edema, injection-site reaction, and alopecia were also observed, but were mild. There was no treatment-related death in this study.

At dose level 1, one of the six patients had DLT. This patient had grade 4 neutropenia and grade 3 anorexia and nausea. Both toxicities began after the administration of TXT on day 15, and the anorexia and nausea resolved 4 days after their onset. But the recovery from neutropenia was delayed for 4 weeks, resulting in the patient's withdrawal from this study. One patient had grade 2 nausea and anorexia, which occurred after the administration of TXT on day 15. The anorexia resolved after 5 days, but the patient rejected continuation of the protocol. The other four patients had no DLT and continued the treatment, and the dose escalation was allowed.

At dose level 2, three of the six patients had DLTs. Two patients had grade 4 neutropenia after the administration of TXT on day 15, and showed a delay of 3 weeks until starting

Table 5. DLT after the first cycle

DLT	Dosage level (No. of patients) 1 (6)	2 (6)
Neutropenia grade 4 for more than 3 days	1	2
Febrile neutropenia grade 3/4	0	0
Thrombopenia grade 4	0	0
Anemia grade 4	0	0
Non-hematologic toxicity grade 3/4	2	0
Anorexia	1	0
Nausea	1	0
Treatment break	1	3

From these results, the MTD of docetaxel/nedaplatin was determined to be 35/80 mg/m^2
Treatment break, break of more than than 2 weeks in therapy

Table 6. Objective responses according to RECIST

Dosage level	No. of patients	No. of patients with each clinical response					Response rate (%)
		CR	PR	SD	PD	NE	
1	6	1	0	2	1	2	16.6
2	6	0	2	3	1	0	33.3
Overall	12	1	2	5	2	2	25

At level 1, two patients were not evaluable because they received only one cycle of the treatment. This study showed a response rate of 25%, including one complete remission

Table 7. Responses of lesions in the esophagus

Dosage level	No. of patients	No. of patients with each clinical response					Response rate (%)
		CR	PR	SD	PD	NE	
1	4	0	1	1	0	2	25
2	1	0	0	1	0	0	0
Overall	5	0	1	2	0	2	20

Recurrences or residues in the esophagus were evaluated by the criteria of the Japanese Society for Esophageal Diseases. The response rate of these lesions was 20%

the second cycle of treatment. One patient had grade 2 thrombocytopenia, and it took 3 weeks to recover to baseline. These three patients were treated at level 1 from the second cycle, and more than two cycles could have been continued without DLTs. The other three patients had no DLT in the first cycle, but all of them had grade 4 neutropenia in the second or third cycle and dosages had to be reduced to dose level 1 to continue the treatment.

From these results, the MTD and RD of TXT/CDGP were determined to be 35/80 mg/m^2 and 30/80 mg/m^2, respectively.

In this dose-escalation study, the efficacy of the therapy was also evaluated according to RECIST, and the results are shown in Table 6. At dose level 1, two patients were not evaluable because they received only one cycle of the treatment. One patient showed complete remission at dose level 1; this patient had multiple lung and mediastinal lymph node metastases and received nine cycles of the therapy. Two patients, who both had multiple lung and mediastinal lymph node metastases, achieved partial responses at dose level 2. In this study, the overall response rate was 25%. Recurrences or residues in the esophagus were also evaluated by the criteria of the Japanese Society for Esophageal Diseases,[23] and the results are shown in Table 7. One patient, who received five cycles of the therapy, achieved a partial response at dose level 1. The response rate of the lesions in the esophagus was 20%.

Discussion

There is no effective recommended treatment for patients with relapsed or refractory esophageal cancer after definitive CRT using CDDP and 5FU. Lordick et al.[15] reported a phase II trial of irinotecan and TXT in CDDP-pretreated relapsed or refractory esophageal cancer and indicated a small likelihood of major remission in this population of patients, but more investigations are essential.

We considered that a combination of TXT and CDGP might be suitable for the treatment of patients resistant to CRT. TXT is a mitotic spindle poison that promotes tubulin polymerization and inhibits the depolymerization of microtubules,[16] and its action mechanisms are quite different from

those of CDDP, CDGP, and 5FU. CDGP has shown superior antitumor activity and less renal and gastrointestinal toxicity as compared with CDDP in some studies.[18,19,24–29] Because there is a possibility that the renal background capacity is decreased in the CDDP-pretreated patient, it may be safe to use CDGP in such patients. It is unclear whether CDGP is cross-resistant to CDDP. It was shown that human leukemia cells resistant to CDDP were sensitive to CDGP,[30] but, on the other hand, CDGP showed cross-resistance to CDDP in lung,[31] ovarian,[32] and cervical[33] cell lines in in vitro studies. However, CDGP showed efficacy in some patients who showed resistance to CDDP-based therapy in the phase II trial we have previously noted[19] and in our preliminary study,[24] and this encouraged us to use CDGP in the treatment of patients resistant to CRT. The DLTs of TXT and CDGP are neutropenia and thrombocytopenia, respectively.[17,19] The toxicity profiles of these two drugs are different, but neutropenia is severe in a 3-week schedule of TXT as the standard regimen. Berstein et al.[20] reported that the administration of TXT in a weekly schedule was effective and well tolerated in women with metastatic breast cancer. Their data showed that decreasing the amount of the drug and shortening the interval between administrations made it possible to maintain efficacy but avoid neutropenia. For these reasons, bi-weekly administration of TXT was used in our study.

The objectives of the present dose escalation study were to determine the MTD, DLT, and RD of combination chemotherapy with TXT and CDGP in patients with relapsed or refractory squamous cell carcinoma of the esophagus after treatment with CDDP, 5FU, and radiation. The MTD was determined to be 35/80 mg/m^2 of TXT/CDGP and the RD was determined to be 30/80 mg/m^2 of TXT/CDGP. DLTs related to the MTD were two grade 4 neutropenias and three treatment breaks longer than 2 weeks due to delays in recovery from these neutropenias, and grade 2 thrombocytopenia. Grade 3 anorexia and nausea and grade 1 renal toxicity were observed, but these non-hematological adverse effects were not related to the DLTs.

Another advantage of this combination therapy was that the treatment was manageable in an outpatient setting. Because CDGP has low renal toxicity and does not require hydration,[18] it takes only 2 or 4 h to complete the administration of TXT and/or CDGP. Most of the adverse events observed in this study were moderate and manageable.

In this study, one-fourth of the patients were responders, including one complete remission lasting for about 8 months. A Japanese phase II study of TXT in advanced or recurrent esophageal cancer reported a response rate of 15.8% in patients previously treated with chemotherapy or CRT.[17] A phase II trial of vinorelbine in metastatic squamous cell esophageal carcinoma reported a response rate of 6% in patients who had prior chemotherapy.[14] A phase II trial of irinotecan plus TXT in CDDP-pretreated relapsed or refractory esophageal cancer reported a response rate of 12.5%.[15] Although our study was a dose-escalation trial and the number of patients was very small, TXT and CDGP showed a response rate of 25% and might be promising agents in the target population.

In conclusion, we conducted a dose-escalation study of combination chemotherapy with TXT and CDGP in patients with relapsed or refractory squamous cell carcinoma of the esophagus pretreated with CDDP, 5FU, and radiation. The RD was determined to be TXT 30 mg/m^2 days 1 and 15 and CDGP 80 mg/m^2 day 1, every 4 weeks. This combination could be a potential second-line treatment for the target population. A phase II study of this combination is ongoing.

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Adjuvant chemotherapy with irinotecan hydrochloride and cisplatin for clear cell carcinoma of the ovary

MASASHI TAKANO[1,13], YOSHIHIRO KIKUCHI[1], NOBUO YAEGASHI[2], MITSUAKI SUZUKI[3], HIROSHI TSUDA[4], SATORU SAGAE[5], YASUHIRO UDAGAWA[6], KAZUO KUZUYA[7], JUNZO KIGAWA[8], SATOSHI TAKEUCHI[9], HITOSHI TSUDA[10], TAKUYA MORIYA[11] and TORU SUGIYAMA[12]

[1]Department of Obstetrics and Gynecology, National Defense Medical College, Tokorozawa, Saitama 359-8513; [2]Department of Obstetrics and Gynecology, Tohoku University, Sendai, Miyagi 980-8574; [3]Department of Obstetrics and Gynecology, Jichi Medical College, Kawachi-gun, Tochigi 329-0498; [4]Department of Obstetrics and Gynecology, Osaka City General Hospital, Osaka, Osaka 534-0021; [5]Department of Obstetrics and Gynecology, Sapporo Medical University, Sapporo, Hokkaido 060-8556; [6]Department of Obstetrics and Gynecology, Fujita Health University, Toyoake, Aichi 470-1192; [7]Department of Gynecology, Aichi Cancer Center Hospital, Nagoya, Aichi 464-8681; [8]Department of Obstetrics and Gynecology, Tottori University, Yonago, Tottori 683-8504; [9]Department of Obstetrics and Gynecology, Kobe National Hospital, Kobe, Hyogo 554-0155; [10]Department of Pathology II, National Defense Medical College, Tokorozawa, Saitama 359-8513; [11]Pathology Laboratory of Central Clinical Facilities, Tohoku University, Sendai, Miyagi 980-8574; [12]Department of Obstetrics and Gynecology, Iwate Medical University, Morioka, Iwate 020-8505, Japan

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Abstract. Clear cell carcinoma (CCC) of the ovary has distinct characteristics showing resistance to conventional platinum-based regimen. Our aim was to evaluate the effects of combination therapy with irinotecan hydrochloride and cisplatin (CPT-P), comparing to regimen with paclitaxel and platinum (TP). We retrospective reviewed 172 patients with complete surgical staging procedures including lymphadenenctomy. Forty-six patients received CPT-P and 126 patients were treated with TP. Survival of the two groups was compared. Between CPT-P group and TP group, there was no significant difference in median age, performance status, FIGO stage, rate of optimal cytoreduction, and follow-up period. There was no significant difference in progression-free survival of patients with stage I tumors (p=0.95) and suboptimally debulked stage II-IV tumors (p=0.92). Although there was no significant difference of overall survival, progression-free survival of CPT-P group was significantly better than that of TP group in optimally debulked stage II-IV (p=0.03). Multiple regression survival analysis revealed CPT-P regimen (p=0.02) and residual tumor diameter (p<0.01) were both independent prognostic factors in stage II-IV tumors. The combination of CPT-P was shown to have a potential therapeutic benefit for advanced CCC of the ovary, especially for cases with optimal debulking surgery. However, this is a limited retrospective study, therefore we recommend that the CPT-P regimen be evaluated in a larger prospective study.

Correspondence to: Dr Masashi Takano, [13]*Present address*: Institute of Reproductive and Developmental Biology (IRDB), Imperial College of London, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK
E-mail: m.takano@imperial.ac.uk

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Introduction

Clear cell carcinoma (CCC) was initially termed as mesonephroid by Schiller in 1939 (1), and since 1973 it was strictly defined by World Health Organization as lesions characterized by clear cells growing in solid/tubular or glandular patterns as well as hobnail cells (2). Since then, many studies have identified the distinctive behavior of the tumors. The most distinctive difference is that patients with CCC of the ovary have poorer prognosis compared with those with other pathological types of epithelial ovarian carcinomas (3,4). Additionally, CCC of the ovary was implied to show resistance to conventional platinum-based chemotherapy (5,6). Recent studies confirmed the evidence in the analysis of measurable CCC patients: response was observed in 11-45% with conventional platinum-based regimen, whereas patients with serous subtype showed a significantly higher response rate of 73-81% (7,8).

Combination with paclitaxel and platinum (TP), recognized as 'Gold standard' regimen for ovarian cancer, is now used to treat patients with all subtypes of ovarian neoplasms, but in measurable CCC cases treated with TP regimen, response was relatively low, ranging from 22 to 56% (9,10; Enomoto T, *et al*, Proc Am Soc Clin Oncol 22: 447, abs. 1797, 2003). One

report showed survival benefit of conventional chemotherapy with paclitaxel and platinum after complete surgery in CCC patients (11). However, the result from large series of CCC patients treated with paclitaxel and platinum showed no survival benefit compared with conventional platinum-based chemotherapy in both early and advanced cases (12).

Irinotecan hydrochloride, a semi-synthetic derivative of camptothecin, has additive and synergic effects in combination with cisplatin *in vitro* (13-15). The combination therapy with irinotecan hydrochloride and cisplatin (CPT-P) was reported to be effective for patients with various solid tumors. Especially, a large clinical trial revealed that CPT-P had significant activity for extensive small-cell lung cancer (16). Additionally, CPT-P has been reported to be effective in first-line and second-line chemotherapy for the treatment of CCC of ovary (17,18). The aim of the present study was to evaluate the therapeutic activity of CPT-P and TP in a retrospective analysis.

Patients and methods

A retrospective review of the patients seen at ten Japanese hospitals from 1 January 1992 to 31 December 2003 was done. Of all the patients treated in those hospitals, the following patients were selected: a) patients who underwent complete surgical staging procedures including hysterectomy, bilateral salpingo-oophorectomy, peritoneal washing, omentectomy, pelvic lymphadenectomy and paraaortic lymphadenenctomy; b) patients whose tumor specimens were confirmed as CCC of the ovary by two pathologists in central pathologic review; c) patients treated with six courses of combination chemotherapy using irinotecan hydrochloride and cisplatin (CPT-P) or six courses of paclitaxel and platinum combination (TP); d) age ≤75 years; e) Eastern Cooperative Oncology Group (ECOG) performance status (PS) of ≤2; f) pretreatment leukocyte count ≥4000/mm^3, platelet count ≥100000/mm^3, hemoglobin ≥9.0 g/dl, serum creatinine <1.5 mg/dl, creatinine clearance ≥60 ml/min, and GOT and GPT less than twice upper limit of normal at the hospitals; g) the study approved by the Committee on Ethics of each hospital.

One cycle of CPT-P regimen consisted of a drip infusion of 50-60 mg/m^2 of cisplatin on day 1 and 50-60 mg/m^2 of CPT-11 on days 1, 8 and 15, and one week off and it was repeated every 4 weeks. TP regimen consisted of a drip infusion of 175-180 mg/m^2 of paclitaxel and 50-75 mg/m^2 of cisplatin or carboplatin (AUC = 5-6).

Response was evaluated with CT or MR images at least every 2 cycles of chemotherapy in the patients with measurable disease. A complete response (CR) was defined as the complete disappearance of all detectable disease for at least 4 weeks. A partial response (PR) was defined as a >50% decrease in tumor size for at least 4 weeks. Stable disease (SD) was defined as the absence of any significant change in measurable lesions for at least 4 weeks. Progressive disease (PD) was defined as the appearance of a new lesion or a >25% increase in tumor size. Serum levels of tumor markers including CA125 were not used for response evaluation of chemotherapy in the present study.

The time to progression was defined as the interval from the date of primary surgery until the date of recurrence or tumor progression. Survival duration was determined as the time from the date of primary surgery until death or the date of last follow-up contact. Kaplan-Meier method was used for calculation of patient survival distribution. The significance of the survival distribution in each group was tested by a generalized Wilcoxon test and the log-rank test. The χ^2 test and Student's t-test for unpaired data were used for statistical analysis. A p-value of <0.05 was considered statistically significant. The Stat View software ver. 5.0 (SAS Institution Inc., Cary, NC, USA) was used to analyze the data.

Results

In total, 46 cases with CPT-P regimen and 126 cases with TP regimen were enrolled in the present retrospective study. TP group included 118 cases treated with paclitaxel and carboplatin and 8 cases treated with paclitaxel and cisplatin. The characteristics of the patients are outlined in Table I. There was no significant difference in median age, performance status, FIGO stage, rate of optimal cytoreduction, and follow-up period between CPT-P group and TP group. Median age of all cases was 53 years (range, 27-75 years). CPT-P group included 24 patients (52%) of stage I, 6 (2%) in stage II, 13 (28%) in stage III and 3 (6%) in stage IV. In TP group, 72 cases (56%) were in stage I, 15 (12%) in stage II, 34 (27%) in stage III and 5 (4%) in stage IV. Optimal cytoreduction (residual tumor diameter <1 cm) with their initial surgery was achieved in 83% (39/46) in CPT-P group and 83% (104/126) in TP group, respectively. The patients with optimal cytoreductive surgery included 6 stage II, 7 stage III and 2 stage IV tumors in CPT-P group and 14 stage II, 16 stage III and 2 stage IV tumors in TP group. Median follow-up period was 28 months in CPT-P group and 27 months in TP group.

All patients who received CPT-P regimen were evaluable for toxicity. In 46 cases who received CPT-P regimen, 35 cases were treated with 50 mg/m^2 of cisplatin and 60 mg/m^2 of CPT-11, 7 cases with 60 mg/m^2 of cisplatin and 60 mg/m^2 of CPT-11, and 4 cases with 50 mg/m^2 of cisplatin and 50 mg/m^2 of CPT-11, respectively. Hematological and non-hematological toxicities in all cases with CPT-P regimen are shown in Table II. The major toxicities were neutropenia and diarrhea. The incidences of grade 3 and 4 neutropenia were 22 and 7%, respectively. Grade 3 nausea and grade 3 diarrhea was observed in 4 and 9% of all cases, respectively. CPT-11 was omitted in 12 (26%) patients on days 8 or 15 because of toxicities, and 20% dose reduction of CPT-11 was documented in 10 (22%) patients.

Response evaluation was assessed in the patients with suboptimal reduction; 22 cases of PT regimen and 7 cases of CPT-P regimen. The patients treated with PT included 1 (5%) CR, 6 (27%) PR, 3 (14%) SD and 12 (55%) PD. CPT-P group consisted of 3 (43%) PR, 2 (29%) SD and 2 (29%) PD, respectively. Response was observed in 7 (32%) of 22 cases in TP group and 3 (43%) of 7 cases in CPT-P group. The rates of non-PD patients were 71% (5 of 7 cases) in CPT-P group and 45% (10 of 22 cases) in TP group, respectively (p=0.047).

Two-year and progression-free and overall survival in stage I tumors was 77 and 92% in CPT-P group and 78 and 94% in TP group, respectively. There were no significant survival differences in progression-free survival (Fig. 1A, p=0.95) and overall survival (p=0.40) in stage I tumors.

Table I. Characteristics of the patients.

	Irinotecan plus cisplatin (%)	Paclitaxel plus platinum (%)	P-value
Patients	46	126	
Median age (years) (range)	52 (32-69)	53 (27-75)	0.85
Performance status			0.17
0	26 (57)	85 (67)	
1, 2	20 (43)	41 (33)	
FIGO stage			0.88
I	24 (52)	72 (57)	
II	6 (13)	15 (12)	
III	13 (28)	34 (27)	
IV	3 (7)	5 (4)	
Primary surgery			0.32
Optimal reduction	39 (85)	104 (83)	
Suboptimal reduction	7 (15)	22 (17)	
Follow-up period (months)			0.43
Median	28	27	
Range	6-83	3-73	

Table II. Hematological and non-hematological toxicity of combination chemotherapy of irinotecan hydrochloride and cisplatin for clear cell carcinoma of ovary.

	Grade of toxicities				
	0 n (%)	1 n (%)	2 n (%)	3 n (%)	4 n (%)
Hematological					
Neutropenia	4 (9)	18 (39)	11 (24)	10 (22)	3 (7)
Anemia	14 (30)	20 (43)	10 (22)	1 (2)	1 (2)
Thrombocytopenia	41 (89)	2 (4)	1 (2)	1 (2)	1 (2)
Non-hematological					
Nausea	1 (2)	23 (50)	20 (43)	2 (4)	-
Fatigue	25 (54)	14 (30)	7 (15)	0 (0)	0 (0)
Diarrhea	12 (26)	21 (46)	9 (20)	4 (9)	0 (0)

Patients with suboptimal cytoreduction surgery included 7 cases of CPT-P group and 22 cases of TP group. In these patients with suboptimal reduction, median survival was 10 months in CPT-P group and 12 months in TP group, and there were no significant differences in progression-free survival (Fig. 1B, p=0.92) and overall survival (p=0.82).

In stage II-IV tumors, optimal reduction at their initial surgery was achieved in 15 (68%) of 22 patients in CPT-P group and 32 (59%) of 54 patients in TP group. Among optimally debulked patients, CPT-P group included 10 cases (67%) of no residual tumor and 5 (33%) cases of tumor diameter <1 cm, and TP group consisted of 24 cases (75%) of no residual tumor and 8 cases (25%) of tumor diameter <1 cm. There was no significant distribution difference of residual tumor diameter in optimally debulked patients between CPT-P and TP group (p=0.55).

Progression-free survival of CPT-P group was significantly better than that of TP group in the group with stage II-IV optimal cytoreduction (Fig. 2, p=0.03). Two-year progression-free survival was 86% in CPT-P group and 44% in TP group, respectively. Median progression-free survival of TP group was 15 months. There was no significant difference in overall

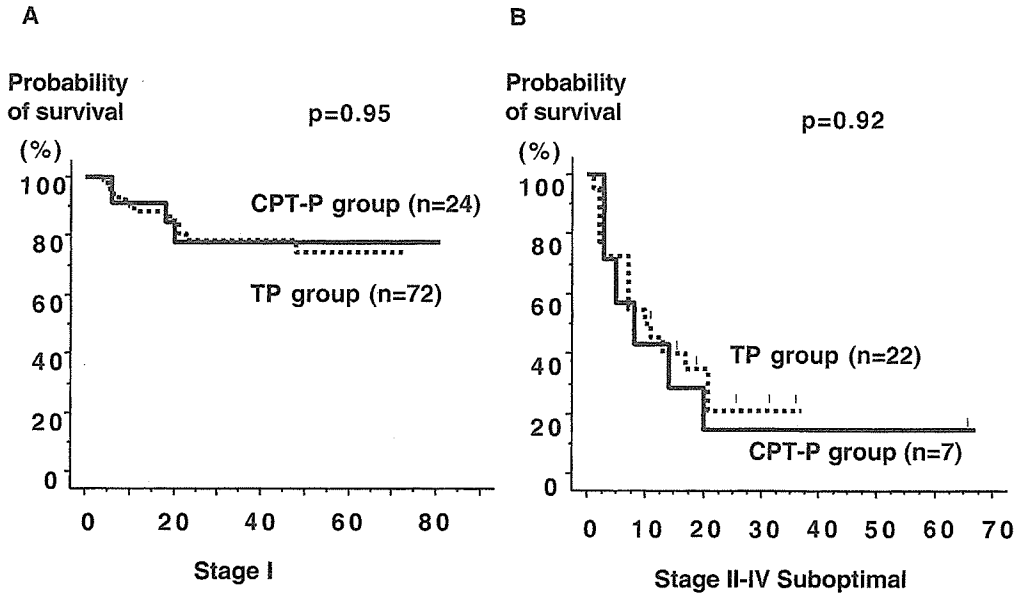

Figure 1. Kaplan-Meier curve comparing the progression-free survival of stage I patients (A) and stage II-IV patients with suboptimal debulking surgery (B) according to adjuvant chemotherapy. No significant difference was observed in patients with stage I (p=0.95), and those with stage II-IV suboptimal debulking patients (p=0.92).

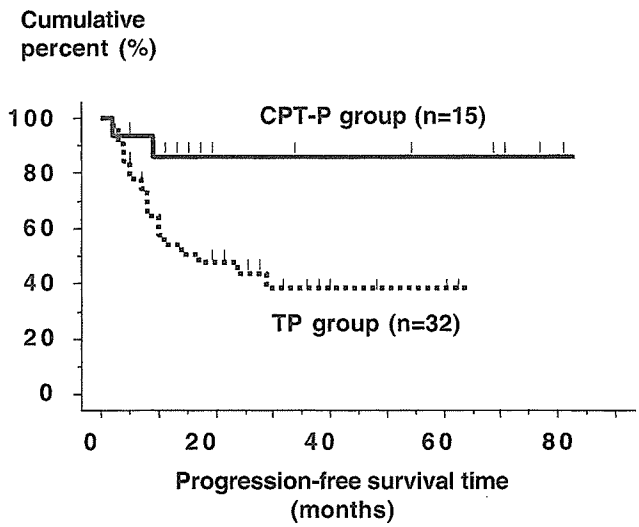

Figure 2. Kaplan-Meier curve comparing the progression-free survival of the stage II-IV patients with optimal debulking surgery according to adjuvant chemotherapy. The survival of the patients with combination with irinotecan hydrochloride and cisplatin (CPT-P) was significantly better than that with paclitaxel and platinum (TP) (p=0.03). Two-year progression-free survival was 86% in CPT-P group and 44% in TP group and median progression-free survival time was 13 months in CPT-P group and 11 months in TP group, respectively.

survival of optimally debulked stage II-IV patients according to two regimens (p=0.14).

In stage II-IV tumors with optimal reduction, two parameters were independent prognostic factors for progression-free survival as determined by multivariate analysis: residual tumor diameter <1 cm (hazard ratio, 3.77; p<0.01); paclitaxel and platinum (hazard ratio, 6.454; p=0.02) (Table III). Age, performance status, and FIGO stage were not significant prognostic factors.

Discussion

In a previous study with CPT-P regimen for ovarian cancer using 70 mg/m^2 of cisplatin on day 1 and 60 mg/m^2 of irinotecan hydrochloride on days 1, 8 and 15, toxicities more than grade 3 was observed in 52-70% for neutropenia and 4-10% for diarrhea (17,19). Japanese large clinical trial using 60 mg/m^2 of cisplatin and 60 mg/m^2 of irinotecan hydrochloride for patients with lung cancer (16) showed high abundance of grade 3-4 toxicities compared with the present study. The lower rates of grade 3-4 toxicities might be explained by a large proportion of patients who received a lower dose (50 mg/m^2) of irinotecan hydrochloride. A variety of single nucleotide polymorphisms (SNPs) in human UDP-glucuronosyltransferase could cause a different toxicity profile in patients treated with irinotecan hydrochloride (20,21). A variety of SNPs in specific genes might cause the disease-specific toxicity profiles. In the present study, the most common grade 3-4 adverse effect was neutropenia observed in 28% of patients, but it was reversible in all cases. Diarrhea more than grade 3 was observed in 4 (9%) patients and it was reversible with conservative treatment. It was suggested that CPT-P regimen was relatively safe and well-tolerated in first-line chemotherapy in CCC patients.

CCC of the ovary has been suggested to lack sensitivity compared to conventional platinum-based chemotherapy (3,4,7). As combination chemotherapy of TP regimen is now established as 'Gold standard' regimen for epithelial ovarian cancer (22,23), the regimen is widely used for all histological sub-types of ovarian tumors. Some reports indicated survival benefit of TP therapy compared with platinum-based chemotherapy in stage I CCC disease combined with complete surgical staging procedures (11) and stage III, IV tumors (9). However, a recent study including 254 patients with complete surgical staging revealed that there was no significant survival

Table III. Multiple regression survival analysis for stage II-IV patients with optimally debulked clear cell carcinoma of the ovary.

Variables	Hazard ratio	95% confidence interval	P-value
Age (years)			0.43
<50	1		
>51	1.55	0.53; 4.41	
Performance status			0.88
0	1		
1, 2	1.08	0.40; 2.90	
FIGO stage			0.33
II	1		
III	1.60	0.32; 8.00	
IV	3.04	0.55; 16.67	
Residual tumor			<0.01
None	1		
<1 cm	3.77	1.42; 9.98	
Chemotherapy			0.02
Irinotecan hydrochloride and cisplatin	1		
Paclitaxel and platinum	6.454	1.40; 29.86	

benefit with TP regimen compared with conventional platinum-based chemotherapy in either early or advance staged patients (12). So far, no anti-cancer agents have been established as a standard regimen for CCC. From view of molecular profiling, CCC is recognized as a completely different category from tumors of other histological subtypes (24,25). These distinct molecular characteristics might support the necessity of another approach for the treatment of CCC of the ovary.

CPT-P regimen, initially introduced as a treatment of platinum-refractory ovarian cancer (19), showed moderate activity for first-line chemotherapy of CCC (17). *In vitro* study suggested that irinotecan hydrochloride as well as paclitaxel was the candidate for anti-neoplastic agents for CCC (26). The present study shows that response rate of CPT-P was almost the same as TP. High abundance of SD patients in CPT-P regimen, acting as 'tumor-dormancy' effects, could possibly lead to better progression-free survival in patients with stage II-IV optimal cytoreductive surgery. The significance of CPT-P regimen was also identified as a favorable prognostic factor with multivariate analysis as well as absence of residual tumor. In addition to the toxicity profile, the efficacy of CPT-P was considered to be satisfactory for the treatment for CCC of the ovary.

Irinotecan hydrochloride was shown to have higher activity than conventional platinum-based regimen for adjuvant setting in combination with mitomycin C (27). Combination chemotherapy including irinotecan hydrochloride was suggested to have a potential anti-tumor effect against CCC of the ovary. CCC of ovary has been reported to have a distinct molecular characteristics as well as a distinctive clinical behavior. Targeting therapy for CCC-specific molecular markers such as hepatocyte nuclear factor-1 ß (28) or ABCF2, a member of ATP-binding cassette gene superfamily (29), could possibly be another strategy for the treatment of CCC of the ovary. Although CPT-P could be a candidate regimen showing some efficacy, a large-scale prospective trial is needed to confirm these observations.

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

Hisatsugu Ohori,[1,2] Hiroyuki Yamakoshi,[3] Masaki Tomizawa,[3] Masatoshi Shibuya,[3] Yuichi Kakudo,[1,2] Atsuko Takahashi,[1] Shin Takahashi,[1] Satoshi Kato,[4] Takao Suzuki,[4] Chikashi Ishioka,[1,2] Yoshiharu Iwabuchi,[3] and Hiroyuki Shibata[1,2]

[1]Department of Clinical Oncology, Institute of Development, Aging and Cancer, [2]Tohoku University Hospital, and [3]Department of Organic Chemistry, Graduate School of Pharmaceutical Science, Tohoku University; and [4]Department of Clinical Oncology, Sendai Medical Center, National Hospital Organization, Sendai, Japan

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]

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Note: Y. Iwabuchi and H. Shibata contributed equally to this work.

Requests for reprints: Hiroyuki Shibata, Department of Clinical Oncology, Institute of Development, Aging and Cancer, Tohoku University, Sendai 980-8575, Japan. Phone: 81-22-717-8547; Fax: 81-22-717-8548. E-mail: hiroyuki@idac.tohoku.ac.jp

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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 oncogenes, 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 G_0-G_1 or G_2-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 oncogenes, 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).[5] 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-disulfophenyl)-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 (IC_{50}) 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 5×10^5 per well, exposed to the derivative compounds at their various concentrations, cultured for 30 hours, collected, and sorted using a FACScan flow cytometer (Beckman Coulter, Inc., Fullerton, CA) as described previously (19). The percentage of each cell fraction corresponding to the sub-G_1, G_0-G_1, S, and G_2-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

[5] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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 all the types of cancer cell lines tested. The IC_{50} values of curcumin ranged from 4.0 to 9.0 μmol/L in the cell lines tested, whereas those of GO-035 ranged from 0.6 to 7.0 μmol/L. GO-035 exhibited 2.3 to 10.0 times more

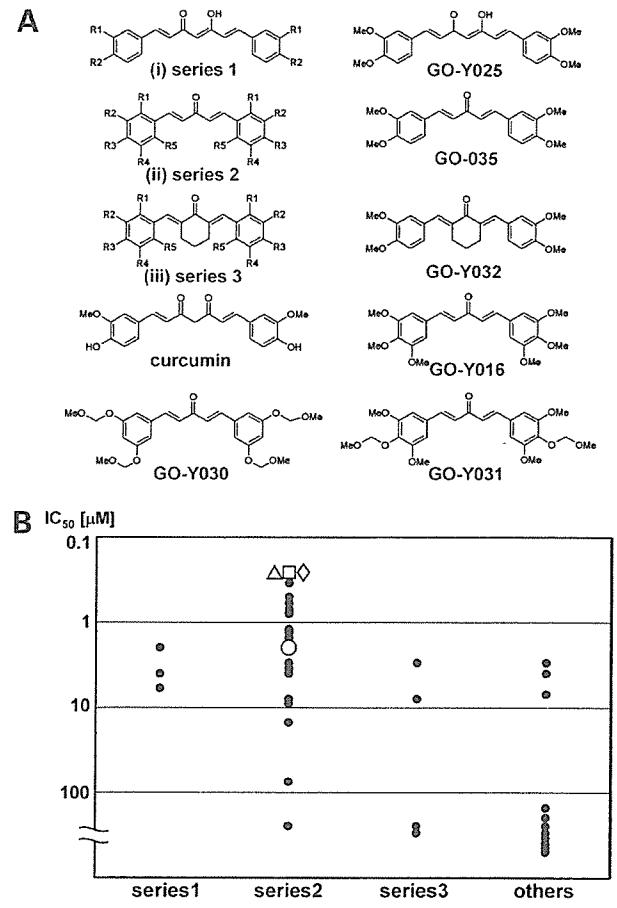

Figure 1. Structures of new curcumin analogues and their abilities to induce cell growth suppression. **A**, common structures of three series of curcumin analogues. **B**, IC_{50} values of the new curcumin analogues against HCT116 cells ($p53^{+/+}$). ○, GO-035; ◇, GO-Y016; □, GO-Y030; Δ, GO-Y031.

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

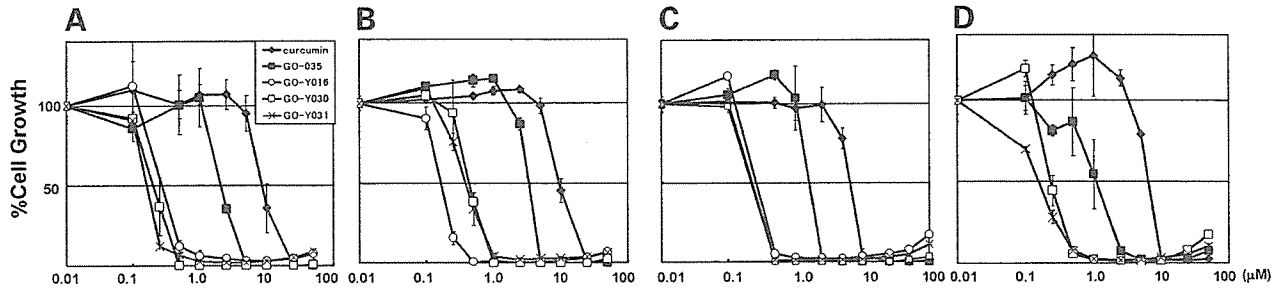

Figure 2. Growth-suppressive potentials of the new curcumin analogues. Growth suppression by curcumin and its analogues. **A**, DLD-1. **B**, SW620. **C**, HCT116 cells ($p53^{+/+}$). **D**, HCT116 cells ($p53^{-/-}$). The percentage of growth in the control cell line treated with 1% DMSO alone was calculated. *Points*, mean of triplicate experiments; *bars*, SD.

during the course of this study suggested that (*a*) the methyl modification of the *p*-hydroxy group relative to the α,β-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).[5] The IC_{50} values of series 2 compounds ranged from 0.25 to over 100 μmol/L (Fig. 1B; Supplementary Table S1).[5] 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 μmol/L (Fig. 2; Supplementary Table S1).[5] 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 μmol/L, except A431 and HepG2, where the IC_{50} value was 2.0 μmol/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.

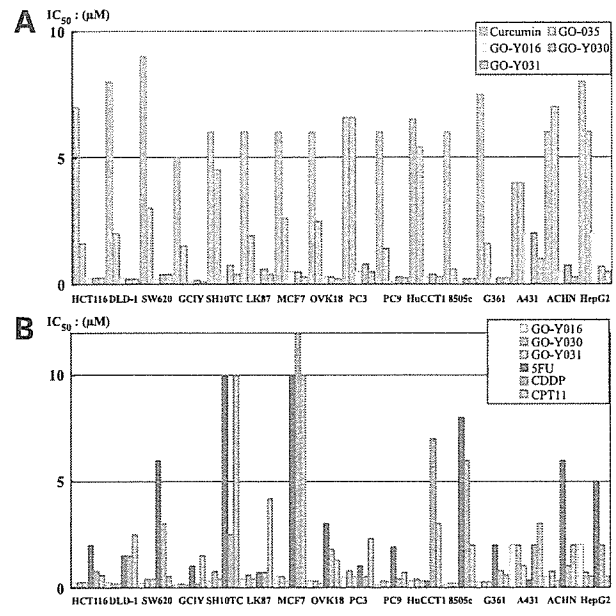

Figure 3. Growth-suppressive abilities of the new curcumin analogues. **A**, growth suppression of various types of cancer cell lines. **B**, comparison of growth suppression with anticancer agents. The origins of cancers are indicated below the name of the cell lines. *Ova*, ovary; *Panc*, pancreas.

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 μmol/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

[IC_{50} 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 IC_{50} ratios (5-FU/diarylpentanoid) in the other 12 cell lines ranged from 6.0 to 80.0, except for the IC_{50} 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 IC_{50} 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 IC_{50} values ranged from 0.16 to 0.7 μmol/L. They were relatively sensitive to CDDP. The IC_{50} 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 IC_{50} values of CPT-11 on 16 cell lines ranged from 0.5 to 10.0 μmol/L. The IC_{50} ratio (CPT-11/diarylpentanoid) 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 G_2-M phase at 20 μmol/L curcumin. Conversely, the cell population at the G_0-G_1 phase was reduced to 7% probably due to the G_2-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 G_2-M phase, and the G_0-G_1 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 G_2-M arrest was observed as seen in 20 μmol/L curcumin. G_2-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-G_1 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-G_1 fraction corresponded to the induction of apoptosis. We suggest that the activities of GO-Y030 and GO-Y031 were reinforced in these two aspects.

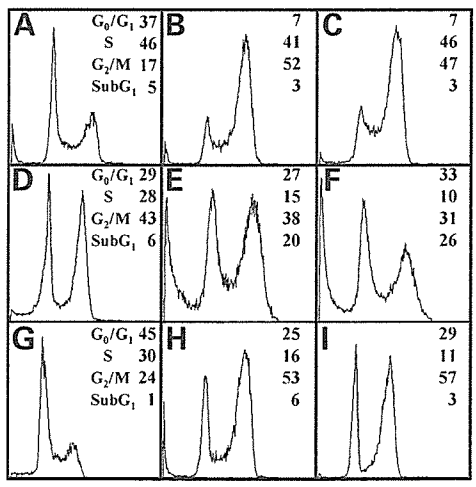

Figure 4. Effects of the new curcumin analogues on cell cycle progression. Cell cycle profile of HCT116 cells ($p53^{+/+}$) treated with curcumin. *Inset,* percentage of cells in G_0-G_1, S, G_2-M, and sub-G_1 phases. **A,** control (1% DMSO). **B,** curcumin (20 μmol/L). **C,** GO-035 (5 μmol/L). **D,** GO-Y016 (1 μmol/L). **E,** GO-Y030 (2 μmol/L). **F,** GO-Y031 (2 μmol/L). **G,** Z-DEVD-fmk (20 μmol/L). **H,** GO-Y030 (2 μmol/L) + Z-DEVD-fmk (20 μmol/L). **I,** GO-Y031 (2 μmol/L) + Z-DEVD-fmk (20 μmol/L).

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 curcumin treatment, respectively (curcumin, 50.69 ± 1.16 arbitrary fluorescent units/min/mg; Supplementary Fig. S3).[5] We found that the new curcumin analogues are slightly superior to curcumin with respect to caspase-3-like activities. However, fluorescence-activated cell sorting analysis clearly indicated that the treatment with caspase-3/caspase-8 inhibitor Z-DEVD-fmk reduced the sub-G_1 fraction to the basal level in either case of GO-Y030 or GO-Y031 (Fig. 4H and I). Conversely, G_2-M arrest was not released in either case when treated with Z-DEVD-fmk (Fig. 4H and I).

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. S5D).[5] To validate the effect on the expression levels of these oncoproteins with the new curcumin analogues, Western blot analyses was 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

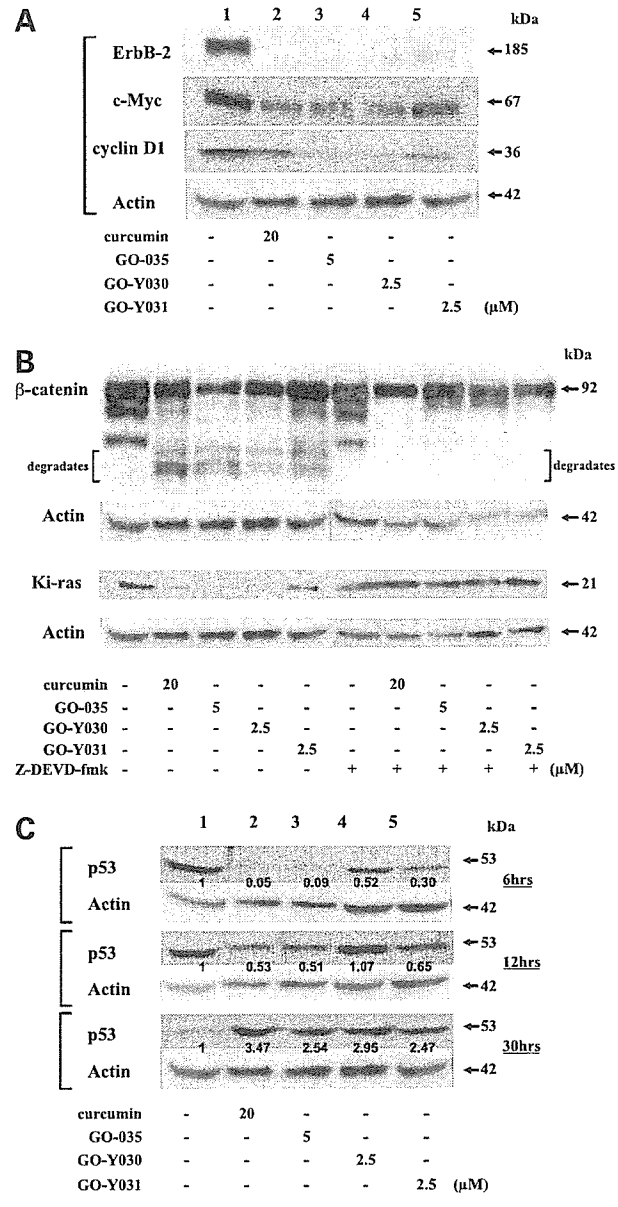

Figure 5. Western blot analyses of the genes affected with the new curcumin analogues. **A,** protein levels of ErbB-2, c-Myc, and cyclin D1 affected with curcumin and its analogues in HCT116. **B,** expression of β-catenin and Ki-ras with curcumin and its analogues. HCT116 cells ($p53^{+/+}$) were treated 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 degradation with curcumin and that caspase-3 plays a crucial role in curcumin-induced β-catenin degradation but neither proteasomal nor lysosomal pathway (10). To examine the involvement of caspase-3 in β-catenin degradation 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 treatment (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 trancriptional, 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 IC_{50} values of GO-Y030 between HCT116 cells ($p53^{+/+}$) and HCT116 cells ($p53^{-/-}$). The IC_{50} 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 diarylpentanoids, 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

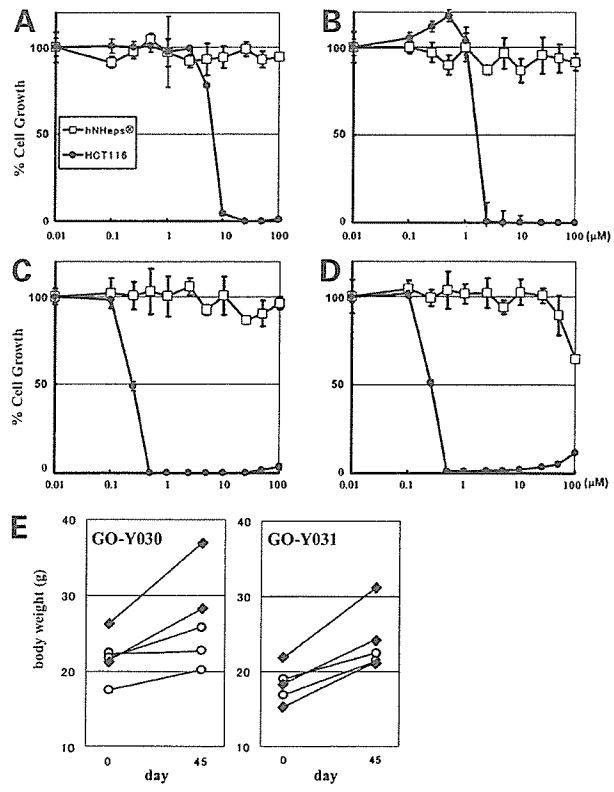

Figure 6. Safety of the new curcumin analogues. Growth-suppressive potential of curcumin analogues was evaluated against the primary hepatocyte (hNHeps; □) compared with HCT116 cells ($p53^{+/+}$; ●). **A**, curcumin. **B**, GO-035. **C**, GO-Y030. **D**, GO-Y031. **E**, safety of the new analogues (GO-Y030 and GO-Y031) *in vivo* was assessed by the body weight.

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 opened 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, G_2-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 overexpression 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.

Acknowledgments

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Original contribution

Differential expression of ABCF2 protein among different histologic types of epithelial ovarian cancer and in clear cell adenocarcinomas of different organs☆

Sadako Nishimura MD, PhD[a], Hiroshi Tsuda MD, PhD[a,f,*], Kiyoshi Ito MD, PhD[b], Toshiko Jobo MD, PhD[c], Nobuo Yaegashi MD, PhD[b], Takeshi Inoue MD, PhD[d], Tamotsu Sudo MD, PhD[e], Ross S. Berkowitz MD[f], Samuel C. Mok PhD[f]

[a]*Department of Obstetrics and Gynecology, Osaka City General Hospital, Miyakojima, Osaka 534-0021, Japan*
[b]*Department of Obstetrics and Gynecology, Tohoku University Graduate School of Medicine, Sendai 980-8574, Japan*
[c]*Department of Obstetrics and Gynecology, School of Medicine Kitasato University, Sagamihara 228-8555, Japan*
[d]*Department of Pathology, Osaka City General Hospital, Miyakojima, Osaka 534-0021, Japan*
[e]*Department of Clinical Research, Hyogo Medical Center for Adults, Akashi 673-8558, Japan*
[f]*Department of Obstetrics, Gynecology and Reproductive Biology, Laboratory of Gynecologic Oncology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA*

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Summary Previously, we reported that ABCF2 protein expression is higher in clear cell than serous histotype of ovarian adenocarcinomas and that its expression correlates with chemoresponse in patients with clear cell ovarian cancer. In this study, we examined ABCF2 protein expression in mucinous, endometrioid, and poorly differentiated type of ovarian adenocarcinomas. In addition, ABCF2 expression was evaluated in clear cell adenocarcinomas derived from different organs. A total of 335 epithelial ovarian cancers, 23 clear cell adenocarcinomas of uterine corpus, and 34 clear cell adenocarcinomas of kidney were included in this study. ABCF2 protein expression was determined by immunohistochemistry. The results showed that cytoplasmic ABCF2 expression was significantly higher in clear cell–type ovarian cancer specimens compared with other types ($P < .0001$). There was a close relationship between nuclear ABCF2 expression levels and age of patients with clear cell ovarian cancer. Multivariate logistic regression model also demonstrated that cytoplasmic ABCF2 expression was associated with clear cell histology (odds ratio, 5.557; 95% confidence interval, 2.694-11.462; $P < .0001$). In addition, both clear cell adenocarcinomas of the ovary and the uterine corpus showed significantly higher levels of ABCF2 expression, compared with those of the clear cell adenocarcinoma

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* Corresponding author. Department of Obstetrics and Gynecology, Osaka City General Hospital, Miyakojima, Osaka 534-0021, Japan.
E-mail address: htsud@s5.dion.ne.jp (H. Tsuda).

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