Anti-invasive effects of curcuminoid compounds from *Curcuma aromatica* Salisb. on murine colon 26-L5 carcinoma cells

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Abstract

Bioassay-directed fractionation of the active chloroform extract from the rhizomes of *Curcuma aromatica* Salisb. (Zingiberaceae) led to the isolation of four main curcuminoid constituents: curcumin (CA-1), demethoxycurcumin (CA-2), 5'-methoxycurcumin (CA-3) and bisdemethoxycurcumin (CA-4). This is the first report to describe the isolation of CA-3 from *C. aromatica*. The chemical structures of these compounds were determined on the basis of spectral analysis and their inhibitory effects on the proliferation, invasion and migration of murine colon 26-L5 adenocarcinoma cells were evaluated *in vitro*. Curcumin and its analogues (CA-2, 3 and 4), at the non-cytotoxic concentration of 10 μM, inhibited the invasive ability of colon 26-L5 cells to the ranges of 22.8, 28.9, 10.3 and 62.0%, respectively. A similar effect of these constituents on the migration of colon 26-L5 cells was also observed. Among these curcuminoids, CA-4 showed the strongest activities, inhibiting both tumor cell invasion and migration in a concentration-dependent manner.

Key words *Curcuma aromatica* Salisb., Zingiberaceae, curcuminoid analogues, metastasis, invasiveness, colon 26-L5 carcinoma cells.

Abbreviations UV, ultra-violet; IR, infra-red; ^{1}H-NMR, ^{1}H-nuclear magnetic resonance; ^{13}C-NMR, ^{13}C-nuclear magnetic resonance; TLC, thin layer chromatrography; AcOEt, ethyl acetate; $CHCl_3$, chloroform; MeOH, methanol; m.p., melting point; MS, mass spectrometry; WST-1, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzenedisulfonate; BSA, bovine serum albumine; DMSO, dimethylsulfoxide.

Introduction

Metastasis is the major cause of morbidity and death for cancer patients. The majority of the patients in the treatment failure group succumb to the direct effect of metastasis or to complications associated with treatment of metastasis.[1] Thus, the development of new drugs for optimal treatment with less resistance and furthermore less complication is required.

Wild turmeric or yellow zedoary (*Curcuma aromatica* Salisb., family Zingiberaceae) is a perennial herb indigenous to and cultivated in Tropical Asia. This plant has no value as a spice but is used in cosmetic formulation and medicines. For medical purposes, the crude drug prepared from its rhizomes has been used as a cholagogue, stomachic, carminative, chloretic, analgesic and sedative product, and also for the treatment of hepatitis, menstrual disorders, epilepsy and skin disorders. It has been recently used as a health food in Japan.[2] Moreover, in Thai and Chinese traditional medicines, it is used for the treatment of several types of cancers such as cervical cancer and liver cancer.[3] This plant is well known as the source of monoterpenoids,[4] sesquiterpenoids[4-8]

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and curcuminoids[9,10] that have been reported to possess antimicrobial,[11,12] antifungal, antioxidant and antitumor activities.[13,14] A previous work reported that β-elemene, a sesquiterpene isolated from *C. aromatica*, markedly prolonged the survival of mice with Ehrlich's ascites tumor and ascitic hepatoma cells *in vitro*.[15,16] However, no information regarding the inhibitory activity of tumor cell invasion and the structure-activity relationship of chemical compounds isolated from this plant has been reported yet.

Several mechanisms for the anti-metastatic effect of curcumin have been reported,[17-22] such as the suppression of invasion of B16F-10 melanoma cells by inhibition of metalloproteinase production[17] or its anti-invasive effect on SK-Hep-1 cells (human hepatocellular carcinoma) that was associated with MMP-9 expression.[18] Curcuminoids also inhibited the angiogenic response stimulated by fibroblast growth factor-2 and the expression of matrix metalloproteinase gelatinase B.[22] In addition, our recent study showed that the combined treatment with curcumin and the anti-cancer drug cis-diamine-dichloroplatinum (CDDP) resulted in a marked inhibition of mediastinal lymph node metastasis of orthotopically implanted LLC (Lewis lung cancer) cells, in addition to the inhibition of tumor growth at the implanted site.[23] Therefore, to extend our investigations on the anti-metastasis activity of curcumin and related compounds, we focused our attention on the anti-invasive effect of curcuminoids isolated from the rhizomes *of C. aromatica* and examined their activities on the *in vitro* proliferation, invasion and migration of the murine colon 26-L5 adenocarcinoma cells.

Materials and Methods

General experimental procedures: All melting points were determined on a Buchi 512 melting point apparatus and are uncorrected. UV absorption spectra were recorded on the double beam spectrophotometer, Hitachi 220 A. IR absorption spectra were obtained using KBr disc on a Shimadzu 440 spectrometer. ^{1}H and ^{13}C-NMR spectra were recorded on a JEOL JNM-FX 500 MHz spectrophotometer in $CDCl_3/CD_3OD$ using tetramethylsilane as an internal standard (δ value, ppm). Mass spectra were measured on a JEOL DX-300/JMA 2000 operating at 70 eV. TLC was performed on pre-coated silica gel 60 F_{254} plate (Merck). Column chromatography was carried out on silica gel (Kieselgel 60 (70-230 mesh), Merck).

Plant materials: Rhizomes of *Curcuma aromatica* were collected in Chiang Mai Province, Thailand in 2000. A voucher specimen was deposited at the Herbarium of the Natural Products Research Section, Research Division, National Cancer Institute, Bangkok, Thailand.

Extraction and isolation: Dried coarse powder of rhizomes (1.5 kg) of *C. aromatica* was extracted successively in a Soxhlet apparatus with *n*-hexane, followed by chloroform and methanol. Concentration of the extracts under reduced pressure afforded crude extracts of *n*-hexane (35.85 g), chloroform (23.79 g) and methanol (32.8 g). All crude extracts were determined for the antiproliferative activity against KB and P388 tumor cells *in vitro*, and the chloroform extract showed the strongest activity.

Purification and identification of curcuminoid compounds: The active chloroform extract (23 g) was subjected to column chromatography on silica gel and eluted with chloroform and methanol by gradient system. Fractions of 75 ml were collected and then combined (*t.l.c.*) to provide 6 fractions (A-F). Fractions B-E, which showed a significant anti-proliferative activity against tumor cell lines, were further purified by repeated column chromatography. Fraction B (5.1 g) was chromatographed on a silica gel column and eluted with chloroform and methanol by gradient system. After recrystallization from *n*-hexane, compound 1 was obtained (orange needles, 1.16 g). Fraction C (4.3 g) was rechromatographed on silica gel column, eluted with chloroform: methanol (19:1) and two compounds were isolated. Re-crystallization of these compounds with *n*-hexane afforded compound 1 (0.907g) and compound 2 (yellow needles, 0.56 g). Fraction D (6.9 g) was rechromatographed on silica gel column, eluted with *n*-hexane: AcOEt (1:1) to give compound 1 (0.21 g), compound 2 (0.92 g) and compound 3 (red powder, 0.14 g). Fraction E (5.8 g) was chromatographed on a silica gel column, eluted with $CHCl_3$: MeOH (9:1) and recrystallized with chloroform to give compound 2 (0.08 g) and compound 4 (yellow needles, 0.82 g).

The identification of these active compounds was confirmed by mixed m.p. and comparison of the spectral

data (UV, IR, ^{1}H and ^{13}C-NMR and MS).

Compound 1: curcumin (CA-1); orange needles from *n*-hexane, m.p. 178-180℃ (lit. 182-183[24]); $C_{21}H_{20}O_6$; MW 368; UV λ_{max} (MeOH) nm (log ε): 261 and 420; IR ν_{max} (KBr) cm^{-1} ; 3450, 2950, 1650, 1600, 1505, 1422, 1275, 1150, 1110, 950, 850 and 800. ^{1}H-NMR ($CDCl_3$) δ : 7.57 (2H, d, J=15.87 Hz, H-1 and H-7), 7.11 (2H, dd, J = 8.24, 1.83 Hz, H-6'and H-6"), 7.03 (2H, d, J=1.83 Hz, H2'and H-2"), 6.92(2H, d, J=8.24 Hz, H-5'and H-5"), 6.46 (2H, d, J=15.87 Hz, H-2 and H-6), 5.78 (1H, s, H-4), 3.93 (6H, s, OMe-3' and 0Me-3"); ^{13}C-NMR ($CDCl_3$) δ : 140.5 (C-1), 121.7 (C-2), 183.2 (C-3), 101.2 (C-4), 183.2 (C-5), 121.7 (C-6), 140.5 (C-7), 127.7 (C-1' and C-1"), 109.6 (C-2' and C-2"), 146.8 (C-3' and C-3"), 147.8 (C-4' and C-4"), 114.8 (C-5' and C-5"), 122.9 (C-6' and C-6"), 55.9 (OMe-3' and OMe-3"); MS m/z (rel. int. %): 368 (M^+, 44), 350 (M^+-H_2O, 40), 177(100).[24-27]

Compound 2: demethoxycurcumin (CA-2); yellow needles from chloroform, m.p. 181-182℃ (lit. 175-177[24]); $C_{20}H_{18}O_5$; MW 338; UV λ_{max} (MeOH) nm(log ε): 250 and 418[1]; IR ν_{max} (KBr) cm^{-1} : 3450, 2820, 1622, 1580, 1560, 1440, 1260, 1150, 1020, 960 and 890; ^{1}H-NMR ($CDCl_3$) δ : 7.52 (1H, d, J=15.87 Hz , H-1), 7.50 (1H, d, J=15.87 Hz , H-7), 7.39 (2H, dd, J=8.54, 1.83 Hz, H-2" and H-6"), 7.04 (1H, d, J=8.54 Hz, H-2'), 7.03 (1H, dd, J=8.54, 1.83 Hz, H-6'), 6.82 (1H, d, J=8.85 Hz, H-5'), 6.79 (2H, dd, J=8.54, 1.83 Hz, H-3"and H-5"), 6.44 (1H, d, J=15.87 Hz, H-2), 6.43 (1H, d, J=15.87 Hz, H-6), 5.80 (1H, s, H-4), 3.87 (3H, s, OMe); ^{13}C NMR ($CDCl_3$) δ : 141.1 (C-1), 121.1 (C-2), 183.1 (C-3), 101.6 (C-4), 183.6 (C-5), 121.4 (C-6), 141.1 (C-7), 127.5 (C-1'), 126.9 (C-1"), 110.5 (C-2'), 130.3 (C-2"), 147.9 (C-3'), 116.1 (C-3"), 148.9 (C-4'), 159.6 (C-4"), 115.6 (C-5'), 116.1 (C-5"), 123.2 (C-6'), 130.3 (C-6"), 55.9 (OMe-3'); MS m/e (% rel. int): 338 (M^+, 29), 320 (M^+-H_2O, 30), 147(100).[24-27]

Compound 3: 5'-methoxycurcumin (CA-3); red powder from chloroform, m.p. 145-146℃; $C_{22}H_{22}O_7$; MW 398; ^{1}H-NMR ($CDCl_3$) δ : 7.58 (1H, d, J=15.87 Hz., H-1), 7.55 (1H, d, J=15.87 Hz, H-7), 7.11 (1H, dd, J=8.00 and 1.83 Hz, H-6"), 7.03 (1H, d, J=1.83 Hz, H-2"), 6.91 (1H, d, J=8.00 Hz, H-5"), 6.78 (1H, s, H-2'), 6.46 (1H, d, J=15.87 Hz, H-2), 6.47 (1H, d, J=15.87 Hz, H-6), 5.79 (1H, s, H-4), 5.86 (1H, brs, OH-4'), 5.76 (1H, brs, OH-4"), 3.93 (3H, s, OMe-3"), 3.92 (6H, s, OMe-3'and OMe-5'); ^{13}C NMR ($CDCl_3$) δ : 140.7 (C-1), 122.0 (C-2), 183.5 (C-3), 101.2 (C-4), 182.8 (C-5), 121.8 (C-6), 140.7 (C-7), 126.6 (C-1'), 127.6(C-1"), 105.1 (C-2'), 109.6 (C-2"), 147.2 (C-3'), 146.8 (C-3"), 137.0 (C-4'), 147.9 (C-4"), 147.2 (C-5'), 114.8 (C-5"), 105.1 (C-6'), 122.8 (C-6"), 55.9 (OMe-3"); 56.3 (2OMe-3' and 5'); MS m/e (% rel. int): 398[28-29]

Compound 4: bisdemethoxycurcumin (CA-4); yellow needles from chloroform, m.p. 222-223℃ (lit. 232-234℃[24]); $C_{19}H_{16}O_4$; MW 308; UV λ_{max} (MeOH) nm: 250 and 480; IR ν_{max} (KBr) cm^{-1} ; 3200-3300, 1622, 1600, 1560, 1510, 1430, 1275, 1231, 1165, 1140, 975 and 830; ^{1}H-NMR (Me_2CO-d_6) δ : 7.61 (d, 2H, J=15.9 Hz, H-1 and H-7), 7.57(d, 4H, J=8.6 Hz, H-2', H-6', H-2" and H-6"), 6.91 (d, 4H, J=8.6 Hz, H-3', H-5', H-3" and H-5"), 5.98 (s, 1H, H-4); ^{13}C-NMR ($CDCl_3$) δ : 141.1 (C-1), 122.3 (C-2), 184.5 (C-3), 101.6 (C-4), 184.5 (C-5), 122.3 (C-6), 141.1 (C-7), 128.2 (C-1' and C-1"), 130.9 (C-2' and C-2"), 116.2 (C-3' and C-3"), 160.9 (C-4' and C-4"), 116.2 (C-5' and C-5"), 130.9 (C-6' and C-6"); MS m/e (rel.int. %): 308 (M^+, 35), 290 (M^+-H_2O, 50), 147 (100).[24-27]

Assay for the anti-metastatic activity against Colon 26-L5 cells

Cell Culture: The murine colon 26-L5 adenocarcinoma cells (colon 26-L5) were maintained as monolayer cultures in RPMI-1640 medium (Nissui Pharm Co. Ltd., Tokyo, Japan) supplemented with 10% fetal calf serum (FCS, Gibco BRL, Life Technologies, NY.), 0.1% sodium bicarbonate and 2 mM glutamine (Wako Pure Chemical Ind., Ltd., Kyoto, Japan) and were incubated at 37℃ in a humidified atmosphere containing 5% CO_2 in air.

Cell Proliferation Assay: Cellular viability in the presence or absence of experimental agents was determined using a WST-1 Cell Counting Kit (Wako Pure Chemicals Ind., Ltd., Japan). Briefly, Colon 26-L5 cells ($1x10^4$) suspended in 100 μl of RPMI-1640 medium containing 0.03% BSA were seeded onto a 96-well culture plate (Costar, Cambridge, MA, U.S.A.). After 24 h of pre-incubation, various concentrations of the isolated compounds were added and then incubated for a further 24 h. At the end of incubation, 10 μl of WST-1 solution were added to each well and then incubated for 4 h. The amount of formazan formed was measured spectrophotometrically at 450 nm using an Immuno Mini NJ-2300

plate reader. Each assay was performed in triplicate. Test compounds were dissolved in DMSO and then diluted with medium. Solutions containing less than 0.1% of DMSO had no cytotoxic effect on the cells. The IC_{50} values were calculated from the mean values ± S.D. of absorbances.

In Vitro Invasion and Migration Assays: Invasion of tumor cell through the reconstituted basement membrane Matrigel was assayed as described previously.[30] Firstly, Transwell cell culture chambers (Costar 3422, Cambridge, MA, U.S.A.) were set up with polyvinylpyrrolidone-free carbonate filters of 8.0 μm pore size (Nucleopore, Pleasanton, U.S.A). The lower surface of the filters was coated with 2 μg of fibronectin (Iwaki Glass Co., Ltd., Japan) and the upper surface was coated with 10 μg of Matrigel (Collaborative Research Inc, Bedford, MA, USA). Colon 26-L5 cells ($2x10^5$ cells/chamber) were suspended in RPMI-1640 (100 μl/chamber) containing 0.1% of bovine serum albumin (BSA) and distinct concentrations of curcuminoid compounds (0, 1, 3 and 10 μM). The cell suspension was then applied to the upper compartment of the chambers and incubated in a 24-well culture plate containing 600 μl of 0.1% BSA-containing medium at 37°C for 24 h. The filters were finally fixed with 30% methanol and then stained with 0.5% crystal violet for 5 min. After gentle rinsing, the cells on the upper surface of the filter were removed by wiping with a cotton swab. The cells that had invaded through the Matrigel and filter were extracted with 30% acetic acid and colorimetrically assessed by measuring its absorbance at 590 nm using an Immuno Mini NJ-2300 plate reader. Each experiment was done in quaduplicate.

The migration assay was performed in a similar procedure to that of the invasion assay, but differed on the non-coating of the filters with Matrigel.

Statistical analysis: All data are expressed as mean values ±S.D. Student's *t*- test for unpaired samples (2-tailed) was used to determine statistic differences that were accepted to be significant when *p* values were lower than 0.05.

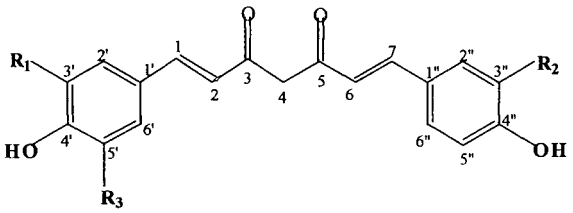
Results

Chemical structures of Curcuminoids isolated from the rhizomes of Curcuma aromatica Salisb.

The chloroform extract, which exhibited a potent anti-proliferative activity against KB and P388 tumor cells *in vitro*, was purified by silica gel column and four curcuminoid compounds were isolated. By the comparison of the spectral data (UV, IR, ^1H and ^{13}C-NMR and MS), these compounds were found to belong to the diarylheptanoids group and were identified as curcumin (CA-1), demethoxycurcumin (CA-2), 5'-methoxycurcumin (CA-3) and bisdemethoxycurcumin (CA-4) (Fig. 1).

Effect of Curcuminoids on Tumor Cell Proliferation

The antiproliferative activity of CA-1, CA-2, CA-3 and CA-4 against colon 26-L5 cells was determined *in vitro* by the WST-1 cytotoxicity assay. As shown in Fig. 2, all compounds at concentrations of less than 10 μM



CA-1: R_1=R_2=OCH_3; R_3=H

CA-2: R_1=OCH_3, R_2=R_3=H

CA-3 : R_1=R_2=R_3=OCH_3

CA-4: R_1=R_2=R_3=H

Fig. 1. Chemical structures of four curcuminoid compounds isolated from *Curcuma aromatica*.

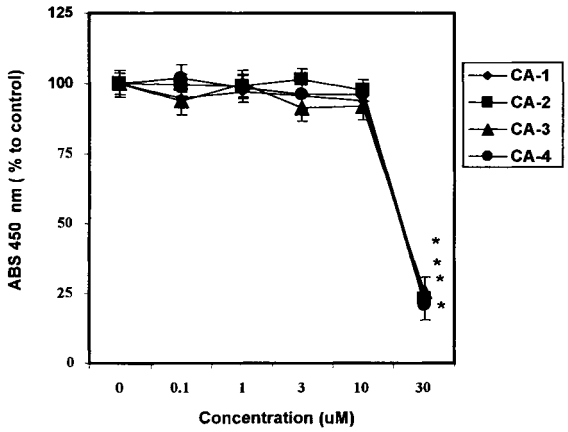


Fig. 2. Effect of curcuminoid compounds on the proliferation of Colon 26-L5 cells.
Colon 26-L5 cells ($1x10^4$ cells/well) were seeded into a 96-well plate in 0.1% BSA-RPMI 1640 medium. After 24 h of incubation, various concentrations of four curcuminoid compounds (◆CA-1, ■CA-2, ▲CA-3, ●CA-4) were added to the cultures and then incubated for an additional 24 h. WST-1 solution (10 μl/well) was added to each well and the plate was incubated at 37°C for 4 h. before termination of the assay. Proliferation was assessed by measuring absorbance of the culture at 450 nm. The data are expressed as mean ± S.D. of triplicate wells. *p<0.01.

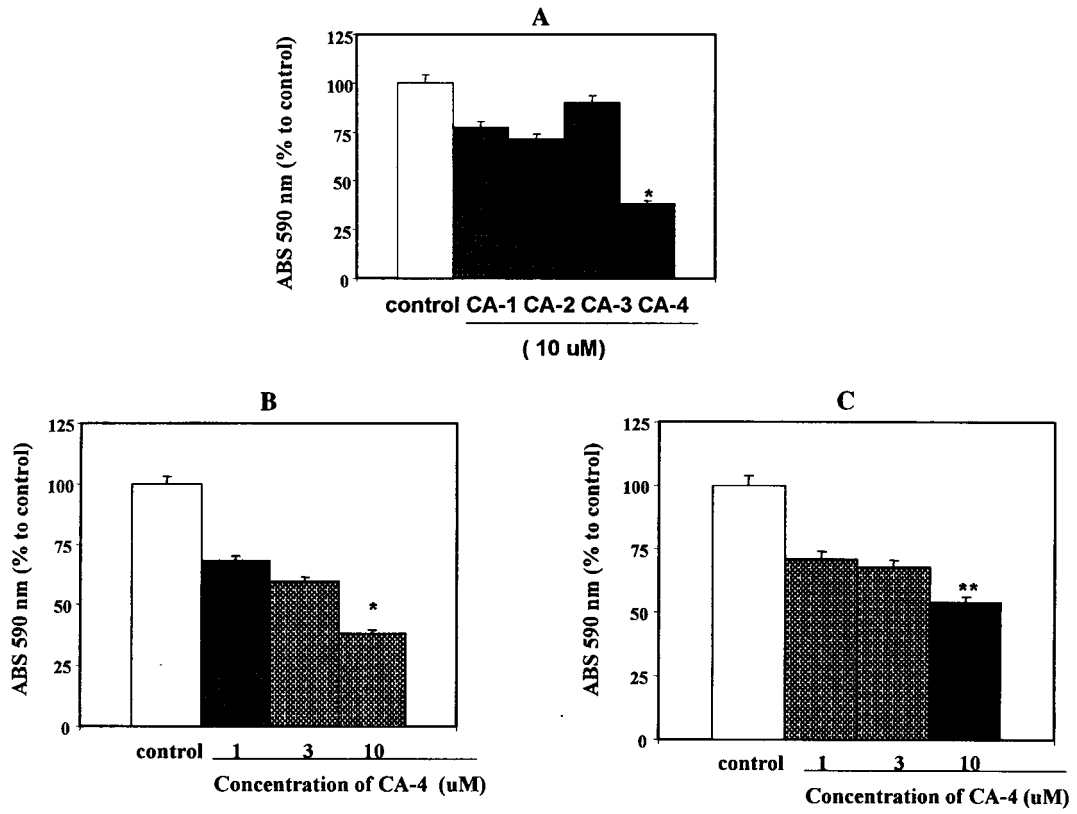


Fig. 3. Effect of curcuminoid compounds on the invasive and migrating properties of Colon 26-L5 cells.
For the invasion assay, Colon 26-L5 cells ($2x10^5$ cells/chamber) were seeded onto filters pre-coated with 10 μg of Matrigel on the upper surface and 2 μg of fibronectin on the lower surface in Transwell chambers in the presence and absence of curcuminoid compounds (10 μM). After 24 h. of incubation, the number of cells that invaded the lower surface was determined by crystal violet staining and colorimetrically assessed by measuring the absorbance at 590 nm. For the migration assay, the filters were not coated with Matrigel. A, Four curcuminoid compounds were tested by the invasion assay; B, Dose-response of CA-4 was determined by the invasion assay; C, Dose-response of CA-4 was determined by the migration assay. The data were expressed as mean ± S.D. of four repeat chambers. *$p<0.01$, **$p<0.05$.

did not show any direct cytotoxicity against tumor cells *in vitro.*

Effect of Curcuminoids on Tumor Cell Invasion and Migration

The effect of curcumin (CA-1) and its analogues (CA-2, 3, 4) on the inhibition of invasion of colon 26-L5 cells through Matrigel/fibronectin- and fibronectin-coated filters were evaluated. Among the compounds, CA-4 at the non-cytotoxic concentration of 10 μM, significantly inhibited the invasion of colon 26-L5 cells as compared with the control (Fig. 3A, $P<0.01$). As shown in Fig 3B and 3C, CA-4 inhibited tumor cell invasion and migration in a concentration-dependent manner.

Discussion

The rhizomes of *Curcuma aromatica* Salisb. were extracted in a Soxhlet apparatus with *n*-hexane, chloroform and methanol as described in the experiment section. The chloroform extract from rhizomes of *C. aromatica* was found to show anti-proliferative activity against KB and P388 cells at IC_{50} values corresponding to 6.8 and 9.0 μg/ml, respectively. The fractionation of this extract by repeated silica gel column chromatography and recrystallization afforded four main curcuminoid constituents: curcumin (CA-1), demethoxycurcumin (CA-2), 5'-methoxycurcumin (CA-3) and bisdemethoxycurcumin (CA-4) (Fig. 1). CA-3 has been found in *Curcuma xanthorrhiza*[28] and *C. zedoaria*,[29] but this is the first report to describe its isolation from *C. aromatica.*

We next investigated the inhibitory effects of CA compounds on the *in vitro* proliferation, invasion and migration of murine colon 26-L5 adenocarcinoma cells, and found that curcumin and its analogues (CA-2 and CA-4) significantly showed antiproliferative activity against colon 26-L5 cells at the concentration of 30 μM (Fig. 2). Moreover, CA-4 at the non-cytotoxic concentration of 10 μM was markedly effective at inhibiting the tumor cell

invasion among the compounds (Fig. 3A). CA-4 also inhibited the migration of tumor cells to fibronectin-coated substrates as well as tumor invasion in a concentration-dependent manner (Fig. 3B and 3C).

Taking into account the inhibitory effect of CA-4 on the invasion assay of colon 26-L5 cells, we suggest that the anti-invasive effect of the curcuminoids investigated in this study might be related with the number of substituted methoxyl or hydroxyl groups in their benzene rings. The greater number of hydroxyl substituents (as seen in CA-4) may contribute to increase this effect, while the greater number of methoxyl substituents (as seen in CA-3) may be associated with a decreased effect. It seems to be in agreement with the known antitumor and antioxidant effects of curcumin that are mainly due to the presence of phenolic groups essential for the free radical scavenging activity, but that are decreased with the presence of methoxyl groups.[28] Some studies on the structure-activity relationships (SAR) of curcuminoid compounds reported that the presence of diketone moiety relates with the inhibitory effect of curcuminoids on the proliferation of human breast cancer cells,[31] or still that hydroxyl groups present at ortho-position on the aromatic rings of curcumin analogs are associated with their inducing activity on Phase 2 detoxification enzymes.[32] To further establish the SAR of compounds CA-1, CA-2, CA-3 and CA-4, the synthesis of derivatives compounds is currently under consideration.

The present study showed that CA-4 had inhibitory effects on the invasion and migration of colon 26-L5 cells without affecting cell growth. Bisdemethoxycurcumin (CA-4) isolated from *C. aromatica* rhizomes may be considered as an inhibitor for tumor invasion and useful for the prevention of liver metastasis of colon cancer without causing toxic effects.

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和文抄録

ショウガ科に属する *Curcuma aromatica* Salisb. の根茎のクロロホルム抽出エキスから，化学構造の明らかな4種のクルクミンおよびその関連化合物：curcumin (CA-1)，demethoxycurcumin (CA-2)，5'-methoxycurcumin (CA-3)，bisdemethoxycurcumin (CA-4) を分離した。これらの化合物を用いてマウス結腸癌細胞 (colon 26-L5) に対する増殖，基底膜への浸潤，細胞運動に及ぼす効果について検討した。クルクミン (CA-1) とその関連化合物 (CA-2, 3 および 4) は，細胞に対して傷害性を示さない10 μMの濃度において，マウス結腸癌細胞の基底膜への浸潤を抑制した（それぞれ22.8, 28.9, 10.3 および 62.0% の抑制率)。この癌細胞の運動能に対しても同様の抑制効果が観察された。これらのクルクミン関連化合物の中で，CA-4 は強い抑制活性を持ち，癌細胞の浸潤および運動能に対して濃度依存的な抑制効果を示した。このように，クルクミン関連化合物の芳香族環の hydroxyl 基および methoxyl 基が癌細胞の浸潤活性の発現と関係している可能性が示唆された。

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