Isolation and Identification of Oleamide as a Growth Inhibitory Compound from the Medium Conditioned by Colon Cancer Cells Treated with Conjugated Linoleic Acid

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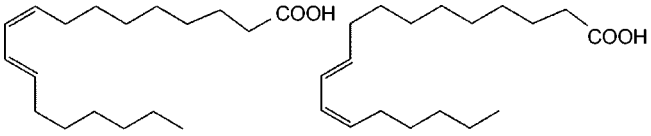
The incidence of colon cancer, one of the most common malignancies in the Western world, is increasing and there is increasing urgency to develop strategies to prevent this disease. With regard to prevention, diet, particularly decreased consumption of fat, has drawn considerable attention in recent years.[1]

Conjugated linoleic acid (CLA) is a naturally occurring substance in food sources, consisting of a mixture of positional and geometric isomers. The predominant isomer of CLA in animal tissues is 9Z,11E (**1**); smaller amounts of 10E,12Z (**2**) also occur. CLA has been shown both in vitro and in animal models to have strong anti-tumor activity.[2] In our previous study, we have utilized a commercial CLA[3] consisting of a mixture of the (9Z,11E) and (10E,12Z)-octadecadienoic acid, to examine whether the CLA mixture inhibits the growth of Caco-2 cells, a human colon cancer cell line.[4] We observed that the CLA mixture inhibited cell growth, and both isomers were incorporated in significant amounts in the membranes of Caco-2 cells.

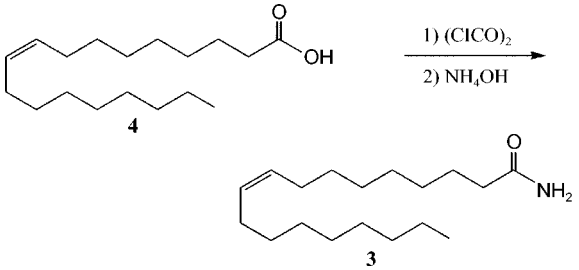


1 **2**

The mechanisms by which CLA exerts the anticarcinogenic effect have not been completely elucidated. Structurally CLA belongs to the family of linoleic acids, the parent compound of arachidonic acid, and thus availability of CLA in the cell seems to affect the endogenous production of arachidonic acid-related compounds and their metabolites. Arachidonic acid is the substrate in the biosynthesis of prostaglandins (PG) and leukotrienes (LT) that control many physiological and pathophysiological consequences of the body including tumorigenesis.[5] We observed that CLA markedly increased material reactive with the antibodies against PGE_2, and LTB_4, determined by a competitive enzyme immunoassay, in a dose-dependent manner.[4] These results suggested that the inhibition of Caco-2 cell proliferation by CLA was attributed to a new compound increased by CLA treatments. We report herein the isolation, identification and synthesis of the newly formed compound from the medium treated with CLA.

To isolate the newly formed compound(s) which reacts with PGE_2 and LTB_4 antisera, cells were incubated in serum-free medium containing 50 μM CLA, and the conditioned media were collected. The conditioned media were acidified with glacial acetic acid, and eicosanoid-like material(s) was extracted with ethyl acetate, concentrated, and separated by column chromatography (CH_3OH-$CHCl_3$ 1 : 9) on silica gel. TLC analysis of the ethyl acetate-extract revealed three spots, and the major spot showing at 0.52 in R_f value (CH_3OH-$CHCl_3$ 1 : 9) was isolated using the flash column chromatography. The amount of other minor two spots on the TLC plate was relatively too small to isolate. The resulting white solid was recrystallized from ethanol and its melting point was determined to be 74-75 °C. High resolution mass spectrometry (HRMS) of this isolated material showed a formula of $C_{18}H_{35}NO$ with apparent mass peak at 281.2714 compared to the expected exact mass of 281.2719 for this formula. The IR (CCl_4) gave two signals at 3519 (asymmetric stretching of N-H) and 3409 (symmetric stretching of N-H) with a strong signal at 1660 (amide C=O) cm^{-1}, indicative of a primary amide. Additionally, the IR (KBr) showed a typical spectrum which indicated the H-bonded N-H stretching of primary amide at 3360 and 3191 cm^{-1}. The ^1H NMR ($CDCl_3$, 400 MHz) showed two NH protons at 5.87 and 5.52 ppm and two olefin protons at 5.35 ppm as a multiplet, and ^{13}C NMR data indicated one carbonyl carbon at 175.9 ppm and two olefin carbons at 130.0 and 129.7 ppm. We previously identified that the compound was oleamide from the spectroscopic data.[4,6]

In order to prove the exact structure and activity of this compound, we synthesized the oleamide **3** from the oleic acid **4**.[7,8] Oxalyl chloride (3 equiv) was added slowly to a solution of oleic acid in CH_2Cl_2 at 0 °C and stirred for 4 h at room temperature. The reaction mixture was concentrated under reduced pressure, cooled to 0 °C, treated with saturated aqueous NH_4OH for 5 min, and extracted with EtOAc.



4 **3**

The organic layer was dried, concentrated and chromatographed (EtOAc : hexane = 1 : 2) to give the oleamide in 75% yield. This authentic oleamide showed the exact match in every respect with the isolated product from the conditioned medium.

To determine whether the synthetic oleamide inhibits Caco-2 cell growth, cells were incubated in serum-free medium in the absence or presence of different concentrations of the oleamide. The concentrations of oleamide used for this experiment were actual levels observed in our previous experiment.[4] In that study Caco-2 cells were cultured in the absence or presence of 50 μM CLA, and six-fold increases in oleamide concentration (600 μM) were observed in the media conditioned by Caco-2 cells treated with CLA compared to those with control (100 μM). As shown in Table 1, the addition of oleamide in culture media resulted in decreased cell number in a concentration dependent manner within 24 hours.

However, oleamide did not inhibit growth of IEC-6 cells, a rat intestinal normal epithelial cell line as shown in Table 2.

Table 1. Effects of synthetic oleamide on cell numbers ($\times 10^{-3}$)

	Oleamide Concentration (μM)			
	0	150	300	600
0 hr	98 ± 2^a	98± 2^a	98± 2^a	98± 2^a
24 hr	138± 2^a	133± 2^a	80± 3^b	61± 4^c
48 hr	216± 7^a	172± 4^b	88± 5^c	70± 4^d

Caco-2 cells were plated in 12-well plates at 80,000 cells/well in DMEM/F12 supplemented with 10% fetal bovine serum. One day later, the monolayers were serum-starved with serum-free DMEM/F12 supplemented with 5 μg/mL transferrin and 5 ng/mL selenium for 24 hours. After serum starvation, cells were incubated in serum-free medium containing 0, 100, 300 or 600 μM oleamide and cell numbers were determined using a hemacytometer. Each value represents the mean ± SEM (n = 6). Values with different superscripts (e.g., a *vs.* b, b *vs.* c, etc.) within each row are statistically different at $p < 0.05$.

Table 2. Effect of oleamide on viable cell numbers in IEC-6 cells

	Oleamide Concentration (μM)			
	0	150	300	600
0 hr	53,280 ± 960^a	53,280 ± 960^a	53,280 ± 960^a	53,280 ± 960^a
24 hr	57,800 ± 2380^a	57,630 ± 680^a	55,760 ± 960^a	58,480 ± 1,700^a
48 hr	79,390 ± 17,00^a	80,240 ± 1,530^a	77,010 ± 1,360^a	73,790 ± 1,020^a

Table 3. Effect of oleic acid on viable cell numbers in Caco-2 cells

	Oleic acid Concentration (μM)			
	0	150	300	600
0 hr	44,200 ± 340^b	44,200 ± 340^b	44,200 ± 340^b	44,200 ± 340^b
24 hr	54,400 ± 1,190^b	70,890 ± 680^b	77,010 ± 850^b	80,240 ± 2,380^b
48 hr	102,000 ± 680^b	129,370 ± 340^b	141,440 ± 2,890^b	162,690 ± 4,250^b

In addition, we have examined if the same concentrations of oleic acid inhibits cell growth and found that oleic acid increased cell number in a dose-dependant manner (Table 3).

In the present study, we have observed that synthetic oleamide decreased Caco-2 cell numbers in a dose-dependent manner. These results with our previous observation that CLA treatments increased oleamide concentrations suggest that oleamide may mediate, at least in part, the growth inhibitory effect of CLA in Caco-2 cells. We propose that oleamide may have potential as a therapeutic and/or preventive agent for colon cancer patients. Future studies are necessary to determine whether oleamide exerts anticarcinogenic effect in experimental animals.

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6. ^{1}H NMR ($CDCl_3$) δ 5.87 (1H, br s, NH), 5.52 (1H, br s, NH), 5.35 (2H, m, HC=CH), 2.22 (2H, t, J = 7 Hz, $COCH_2$), 2.03 (4H, m, CH_2C=CCH_2), 1.63 (2H, quintet, J = 7 Hz), 1.36-1.24 (20H), 0.88 (3H, br t, J = 7 Hz, CH_3); ^{13}C NMR ($CDCl_3$) δ 175.9 (C=O), 130.0 (C=C), 129.7 (C=C), 36.0, 31.9, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 29.1, 29.0, 27.2, 27.1, 25.5, 22.6, 14.1; IR (KBr): 3360 (H-bonded asymmetrical stretch of N-H), 3191 (H-bonded symmetrical stretch of N-H), 3007 (=C-H stretch), 1660 (C=O stretch), 1631 (C=C stretch and N-H bend), 1468, 1423, 1134 (C-N stretch), 701 cm^{-1}; Ms m/z 281 (M^+), 126, 98, 81, 72, 59 (base) (Found 281.2714. $C_{18}H_{35}NO$ requires M, 281.2719); R_f 0.52 (MeOH : $CHCl_3$ = 1 : 9), m.p. (Recrystallized from ethanol) 74-75 °C.
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