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Comparative evaluation of growth inhibitory effect of stereo isomers of fucoxanthin in human cancer cell lines
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Abstract

Inhibitory effects of geometrical isomers of fucoxanthin - characteristic carotenoid of brown seaweeds - on the growth of various cancer cells and on human leukemia (HL-60), colon cancer (Caco-2) and prostate cancer cells (PC-3 and LNCap) was comparatively evaluated. All trans fucoxanthin was the major geometrical isomer (~88\%) found in the fresh brown seaweed (\textit{Undaria pinnatifida}) apart from a small amount of 13-cis and 13’-cis isomers (~9\%). Incubation of the fucoxanthin isomeric mixtures, all trans fucoxanthin with a small amount of 13-cis and 13’-cis isomers, produced 9’-cis isomer (5\%) and increased the contents of 13-cis, and 13’-cis isomers (27\%). The antiproliferative effect of the mixture of 13-cis and 13’-cis isomers was stronger than all other geometrical isomers evaluated in the study. The inhibition of growth of HL-60 cells was higher in case of 13’-cis isomer followed by 13-cis and all-trans isomers. The potent inhibitory effect of 13-cis and 13’-cis fucoxanthin on HL-60 cells and Caco-2 cells could possibly be due to their higher apoptosis inducing activity.

\textit{Keywords:} Fucoxanthin; Isomer specificity; Human cancer cells; Apoptosis
1. Introduction

Dietary carotenoids, including non-pro-vitamin-A carotenoids, are considered to play a role in the prevention of common chronic diseases such as cardiovascular disease, age related macular degeneration and carcinogenesis (Cooper et al. 1999a, b). Epidemiological studies have established a positive correlation between carotenoid consumption and a reduced risk of cancer (Riboli & Norat, 2003; Willett, 2001). β-Carotene is the commonest carotenoid found mainly in vegetable, fruits and other plants. Apart from its ability to convert into vitamin-A β-Carotene is also used as a “gold standard” model to study the relationship between carotenoid intake and cancer prevention over several decades. On the contrary evidence suggests increased incidence of lung cancer in smokers taking pharmacological levels of β-Carotene (Beta Carotene Cancer Prevention Study Group, 1994); and, increased mortality due to cardiovascular disease in smokers, former smokers and asbestos exposed individuals in the β-carotene and retinol efficiency trial (Omenn, et al., 1996). These contra effects have lead researchers to focus on different carotenoids as alternatives.

Lycopene is one of the major carotenoids in the Western diet. It accounts for about 50% of carotenoids in the serum of Western people (Gerster, 1997; Krinsky et al., 1990). Epidemiological investigations have revealed an inverse relationship between serum lycopene levels and increased risk of prostate cancer (Hadley et al., 2002). It is reported that between 10 and 20 cis isomers are typically observed in human blood and account for the majority of lycopene in serum (Hadley et al., 2002). In human prostate tissue, the cis isomer content is higher (80 to 90%) than in other tissues and blood (Clinton et al., 1996). Bouileau et al. (1999) have reported that cis isomers of lycopene are more bioavailable than trans lycopene probably due to higher solubility of cis isomers in bile acid micelles that results in preferential incorporation into chylomicrons.

Isomerization is a common feature of carotenoids due to the presence of conjugated double bonds in their structures. Generally the trans isomers of carotenoids are more common in foods and are more stable as compared to their cis counterparts. Although much work has been done on the physiological effects of trans isomers, very little is known about the biological significance of cis isomers and their role in human health. Factors that contribute to cis-trans isomerization are light, thermal energy, chemical reactions and interaction with biological molecules such as proteins. Recently significance and role of cis and trans lycopene in different biological reactivities are being researched. However, biological significance of geometrical isomers of other carotenoids have received less attention.

Fucoxanthin is the most abundant of all carotenoids mainly found in brown
seaweeds and accounts for >10% of the estimated total natural production of carotenoids (Matsuno, 2001). Fucoxanthin has an unusual allenic bond and 5,6-monoepoxide in its molecule (Fig. 1) that contribute to its unique structure. Fucoxanthin is reported to be very effective in inducing apoptosis in human leukemia (Hosokawa et al., 1999; Kotake-Nara et al., 2005) and colon cancer (Hosokawa et al., 2004; Das et al., 2005) cells. Further, neoxanthin and fucoxanthin - from among 15 carotenoids of food origin - exhibited the most potent growth retarding activity in case of different human prostate cancer cells (PC-3, DU 145 and LNCap) (Kotake-Nara et al., 2001). The higher anticancerous activity of neoxanthin and fucoxanthin was attributed to their characteristic chemical structure. However, information is lacking on the differences in physiological effects of cis and trans isomers of any of these aforementioned carotenoids. Against this backdrop, the present study was conducted to evaluate the differences, if any, in the inhibitory effect of cis and trans isomers of fucoxanthin on various cancer cell lines.

2. Materials and methods

2.1 General

Dried brown alga (Undaria pinnatifida) used for isolation of fucoxanthin and its isomers was procured from the local market. RPMI 1640 medium and antibiotics were purchased from Gibco (Grand island, NY, USA). Fetal bovine serum (FBS) was obtained from ThermoTrace (Melbourne, Australia). Monoclonal antibodies against Bcl-2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Human leukemia cells (HL-60), colon cancer cells (Caco-2), and prostate cancer cells (PC-3 and LNCap) were obtained from the Dainippon Pharma Co. (Osaka, Japan). All the solvents used for HPLC analysis were of HPLC grade and other solvents used for extraction were of analytical grade unless otherwise mentioned.

2.2. Fucoxanthin separation from algal lipids

Fucoxanthin was isolated from the brown alga, Undaria pinnatifida, as follows. Dried U. pinnatifida purchased from a market was ground to a powder in an electric mill, and extracted with two volumes (v/w) of acetone for one night. Fucoxanthin was then separated from the extracts by a silica gel column chromatography (CC) with acetone:n-hexane (1:4, v/v) as eluent. HPLC analysis of fucoxanthin (a mixture of all-trans and 13-cis + 13’-cis isomers) obtained by silica-gel CC indicated the purity of fucoxanthin to be >97% (Fig. 2 (A)). HPLC analysis was performed at 30 °C using two serially connected reverse-phase (RP) columns (Develosil C30 UG-5, 250 x 4.6 mm i.d., 5.0 µm particle size, Nomura Chem. Co., Seto, Aichi, Japan) with a mixture of methanol
and acetonitrile (70:30, v/v) as the mobile phase at a flow rate of 1.0 mL/min. The eluent was monitored at 450 nm with a spectrophotometric detector (Hitachi L-2400, Hitachi Seisakusho Co., Tokyo, Japan).

2.3. Isomerization of fucoxanthin

The fucoxanthin obtained from seaweed lipids as described above was placed at 5°C for 2 months in the dark to isomerize with minimum decomposition. Increase in contents of 9'-cis isomer and a mixture of 13- and 13'-cis isomers was evident after the incubation period as noticed by RP-HPLC analysis (Fig. 2 (B)). HPLC conditions were the same as those described in the previous section. Isomeric mixture of 13- and 13'-cis fucoxanthin was collected and subjected to normal phase HPLC using three serially connected silica columns (Mightsil Si-60, 250 x 4.6 mm i.d., 5.0 µm particle size, Japan) with the solvent mixture of n-hexane/acetone/diethyl ether (75:20:5, v/v/v) at flow rate of 1.5 mL/min as the mobile phase (Fig. 3). All the peaks were monitored at 450 nm to collect each peak individually. Geometrical isomers of fucoxanthin (all trans, 9-cis, 13-cis, and 13'-cis) were characterized by NMR. Each fucoxanthin isomer isolated by HPLC (purity>99%) was dissolved in CDCl3. The 1D-NMR (1H) and 2D-NMR (1H-1H COSY, 1H-1H NOESY) spectra of the isomers were recorded on a Bruker AVANCE600 (Rheinstetten, Germany). These isomers whose identity was established by NMR were used for assaying the antiproliferative activity of individual isomers on various human cancer cell lines.

2.3. Cell lines and cell culture

Caco-2 cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acid, 100 U/mL penicillin and 100 µg/mL streptomycin. HL-60, PC-3, and LNCap cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin, respectively. Cell cultures were maintained in a humidified atmosphere of 95% air and 5% CO2 at 37°C.

2.4. Cell viability assay

HL-60 cells (1x10^5 cells/mL) were cultured in 6-well plates with 5 mL of RPMI-1640 medium per well. After 24 h pre-incubation, individual fucoxanthin isomers were added into the culture medium. The isomers were dissolved in ethanol and the ethanol concentration was adjusted in such a way that its concentration did not exceed 0.1% of the culture medium. Viable cell numbers of HL-60 cell were assessed after 24h and 48 hours of incubation by trypan blue dye exclusion method (Hosokawa et al., 1999).

Caco-2, PC-3 and LNCap cells (2x10^3 cells/well) were cultured in 96-well
microplate with 100 µL of respective medium per well for 24 h. Each isomer of fucoxanthin was dissolved in ethanol and then prepared 5% ethanol solution using medium. Ten microliter of fucoxanthin solution (5% ethanol solution) was added into cultured medium. Cell viability was assessed using WST-1 regent (Wako Pure Chemical, Osaka, Japan). This assay is based on the principle that mitochondrial dehydrogenase of viable cells cleave the tetrazolium salt WST-1 to form formazan dye (Hosokawa et al., 2004). A number of viable cells was measured colorimetrically and expressed as a percentage of the viability in relation to control cultures.

2.5. Measurement of DNA fragmentation

Level of fragmented DNA was measured as an indicator of apoptotic cell death. This was performed using a commercial kit (Cell Death Detection ELISA, Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions. The assay is based on a quantitative sandwich enzyme immunoassay to detect the histone-associated DNA fragments produced during apoptosis. Cell culture conditions were the same as in WST-1 assay.

2.6. Western blot analysis

HL-60 cells (1.5x10⁶ cells) were cultivated in 150 mm tissue culture dish for 24 h followed by addition of ethanolic solution of fucoxanthin isomers into the cultured medium. The final ethanol concentration in the medium was below 0.1% (v/v). Post incubation, adherent cells were trypsinized and washed three times with phosphate buffered saline (PBS). Pellet was then scraped into cold RIPA buffer (pH 7.4) containing 20 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 0.1 mg/mL phenylmethylsulphonyl fluoride, 50 µg/ml aprotinin and 1mM Na₃VO₄. Further, cell lysates were centrifuged at 4°C, 15,000 rpm for 20 min. The proteins in supernatant (40 µg protein/lane) were separated electrophoretically on a 10% SDS-polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride membrane, and blocked with TBS-T (20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.1 % Tween 20) containing 5 % non-fat dry milk for one hour at room temperature. Later, membrane was incubated with anti-human Bcl-2 antibody for one hour at room temperature. After washing, the membranes were incubated with a secondary antibody, anti-mouse IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for one hour at room temperature. Finally, the membrane was treated with the reagents in the chemiluminescence detection kit (ECL system, Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer’s instructions. Actin was used as the control with human actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).
2.7. Incorporation of geometrical isomers of fucoxanthin into HL-60 cells.

HL-60 cells (1.5x10^5 cells) were cultured in 25 mL tissue culture dish with 5 mL medium per dish for 24 h and fucoxanthin isomers then added into cultured medium as ethanol solution. The final ethanol concentration was below 0.1% (v/v). After 6 h, 12 h, 24 h, and 48 h of incubation, cells were washed three times with PBS. Cells were dispersed in a 3 mL PBS and the cell number was counted. Total lipids (TL) were extracted from the cells with chloroform/methanol (2:1, v/v). For the quantification of all-trans fucoxanthin and a mixture of 13-cis and 13’-cis fucoxanthin in HL-6 cells, the extracted lipids was dissolved in 1 mL methanol and acetonitrile (70:30 v/v), and then 10 µL of the solution was injected to HPLC. HPLC analysis was performed as described elsewhere. Fucoxanthin isomers were quantified using calibration curves for all-trans fucoxanthin and a mixture of 13-cis and 13’-cis fucoxanthin.

2.8. Statistical analysis

Data are expressed as means ± SD. Significant difference between multiple groups was determined by ANOVA. Differences with P<0.01 or P<0.05 were considered significant. In case of significance, mean separation was achieved by either Scheffe’s F-test or Tukey’s test. Analysis between two groups was determined using the unpaired Student t test.

3. Results

3.1. Separation and establishment of identities of fucoxanthin isomers

All-trans fucoxanthin (~88%) was the major geometrical isomer of fucoxanthin obtained through silicic acid column chromatography of algal lipids while a mixture of 13-cis and 13’-cis isomers (~9%) constituted the next major isomers (Fig. 2 (A)). Upon storage of all-trans isomer rich fucoxanthin in a refrigerator for 2 months at 5°C in the dark, a significant amount of 9’-cis isomer was observed and contents of 13-cis and 13’-cis isomers increased (Fig. 2 (B)). Identity of each fucoxanthin isomer (Fig. 1) was established by NMR analysis. Chemical shift values of 1H-NMR for each fucoxanthin isomer corroborated with previously reported values (Haugan et al. 1992). Furthermore, identities were confirmed by strong NOE cross peaks between H-8’ and H-11’ for 9’-cis isomer; H-12 and H-15 for 13-cis isomer; H-12’ and H-15’ for 13’-cis isomer; and, by NOESY spectrum between H-10’ and Me-19’ for 9’-cis isomer; H-14 and Me-20 for 13-cis isomer; H-14’ and Me-20’ for 13’-cis isomer, respectively.

3.2. Effect of geometrical isomers of fucoxanthin on HL-60 cells

All fucoxanthin isomers decreased the viability of HL-60 cells significantly (p<0.05 or 0.01) as compared to control (Fig. 4). The viability of HL-60 cells treated with
mixture of 13-cis and 13’-cis fucoxanthin was significantly less than that observed in case of all-trans and 9’-cis fucoxanthin (Fig. 4). Thus, a further investigation on the effects of 13-cis and 13’-cis fucoxanthin on HL-60 cells was conducted after isolation of both isomers (Fig. 5). The viable HL-60 cells treated with 13’-cis fucoxanthin for 24 h significantly decreased as compared to control as well as the other two isomers. After 48 h incubation, the number of viable cells treated with all fucoxanthin isomers were significantly less than that of control. Further, significant difference was observed between those treated with all-trans fucoxanthin and 13-cis or 13’-cis fucoxanthin (Fig. 5).

3.3. Effect of geometrical isomers of fucoxanthin on Caco-2, PC-3, and LNCap cells

All-trans fucoxanthin and a mixture of 13-cis and 13’-cis fucoxanthin also exhibited remarkable antiproliferative effects on Caco-2 cells, PC-3 cells, and LNCap cells (Fig. 6). Time dependent viability of treated cancer cell lines was observed in case of fucoxanthin isomers. Among all the cancer cell lines, viability of cells treated with mixture of 13-cis and 13’-cis fucoxanthin was significantly less than those treated with all-trans fucoxanthin. Fig. 7 presents the anti-proliferative effect of all-trans fucoxanthin, 13-cis fucoxanthin and 13’-cis in Caco-2 cells. Although all fucoxanthin isomers caused a significant reduction in cell viability as compared to control, 13’-cis fucoxanthin exhibited the strongest inhibitory effect on the cell growth, followed by 13-cis fucoxanthin and all-trans fucoxanthin, in that order.

3.4. Apoptosis induction in HL-60 and Caco-2 cells by geometrical isomers of fucoxanthin

The morphological examination of HL-60 and Caco-2 cells treated with geometrical isomers of fucoxanthin revealed a diminished size and rounded shape (data not shown). The cell membrane had shrunk with a condensed cytoplasm. The morphological appearance of both cancer cells treated with fucoxanthin isomer has the properties typical of apoptotic cells. To prove that apoptosis induction occurred due to fucoxanthin, DNA fragmentation in HL-60 and Caco-2 cells was measured as an indicator of apoptosis by quantitative sandwich ELISA using an anti-histone antibody and an anti-DNA antibody. When HL-60 cells were incubated in the culture medium with 10 µM each of all-trans fucoxanthin and a mixture of 13-cis and 13’-cis fucoxanthin for 6 h, relative DNA fragmentation increased significantly as compared to control (Fig. 8 (A)). The relative fragmentation was more enhanced (2.5-fold compared to that in control cells) in case of cells treated with mixture of 13-cis and 13’-cis fucoxanthin just after 12 h incubation. Similarly the mixture of 13-cis and 13’-cis fucoxanthin also induced significant DNA fragmentation in Caco-2 cells after 24 h of
incubation Fig. 8 (B)). However, the effect of all-trans fucoxanthin on DNA fragmentation was not significant even after 24 h of incubation.

3.5. Effect of geometrical isomers of fucoxanthin on proteins involved in apoptosis

In an attempt to explore the effects of geometrical isomers of fucoxanthin on apoptosis regulating proteins, we examined expression of apoptosis suppressing protein Bcl-2. Fig. 9 shows the results of immuno-histochemical analysis of Bcl-2 expression in HL-60 cells incubated with all-trans fucoxanthin and a mixture of 13-cis and 13’-cis fucoxanthin for 24 h and 48 h. Treatment with these fucoxanthin isomers reduced the expression of Bcl-2 protein. This effect was stronger in case of the mixture of 13-cis and 13’-cis fucoxanthin. Hence, it is possible that one mechanism by which cis-isomers induce apoptosis is by suppressing proteins that reduce apoptosis.

3.6. Incorporation of geometrical isomers of fucoxanthin into HL-60 cells and their isomerization.

When HL-60 cells were incubated with different concentrations of all-trans fucoxanthin (Fig. 10 (A) and (B)), quick uptake of fucoxanthin into the cellular lipids was observed as evidenced by their increased content after 24 h incubation followed by a decrease later. However, the uptake of both cis-isomers of fucoxanthin was low. All-trans fucoxanthin was also detected in the extracted lipids of HL-60 cells incubated with a mixture of 13-cis and 13’-cis fucoxanthin (Fig. 10 (C) and (D)). The results in Fig. 10 shows that cis fucoxanthin easily isomerized to all-trans form, while isomerization of all-trans fucoxanthin to cis isomers was hardly occurred. Interestingly, only a little cis isomerization was found during incubation of all-trans fucoxanthin in the culture medium (Fig. 11 (A)), whereas cis fucoxanthin rapidly isomerized to all-trans fucoxanthin 6 h after incubation (Fig. 11 (B)). Thus it can be hypothesized that inspite of cis-isomers of fucoxanthin being stronger in anti-proliferative activity, the mechanism by which they act is by converting themselves into all-trans form. In addition the ease with which all-trans form is taken up could be the reason for this bioconversion of cis to trans form.
4. Discussion

Carotenoids originating from foodshave been reported to inhibit the growth of human cancer cells (Elliott, 2005). Fucoxanthin has been reported to show stronger antiproliferative effect on human leukemia cell (Hosokawa et al., 1999), prostate cancer cells (Kotake-Nara et al., 2005; Kotake-Nara et al., 2001) and colon cancer cells (Hosokawa et al., 2004), as compared to other carotenoids except for neoxanthin. This higher activity of fucoxanthin and neoxanthin has been attributed to their unique structure. Fucoxanthin extracted from wakame (Undaria pinnatifida) completely inhibits the proliferation of the HL-60 cancer cells at concentrations as low as 22.6 µM (Hosokawa et al. 1999).

All-trans fucoxanthin is the major isomer of fucoxanthin found in natural sources, especially in brown seaweeds. In principal, each double bond in the polyene chain of a carotenoid can exist in two configurations, designated as trans or cis, depending on the disposition of substituent groups. The presence of a cis double bond creates greater steric hindrance between nearby hydrogen atoms and/or methyl groups, so that cis isomers are generally less stable thermodynamically than trans form. Most carotenoids, therefore, occur in nature predominantly or entirely in the all-trans form (Fig. 1 (A)). With some double bonds, i.e., those that bear three substituents, the steric hindrance is relatively small so that isomers with cis double bonds in these positions are relatively easily formed and relatively stable (e.g., 9-cis, 9'-cis, 13-cis, and 13'-cis) (Fig. 1 (B)). Therefore, 9'-cis, 13-cis, and 13'-cis isomers were formed as main isomers during the incubation of all-trans fucoxanthin.

The present study reiterates the anti-proliferative effect of fucoxanthin as reported by our group (Hosokawa et al., 1999 and 2004) by establishing the fact that naturally derived fucoxanthin isomers show inhibitory effect on the growth of various cancer cell lines.

Comparison between positional and geometrical isomers of fucoxanthin showed that the anti-proliferative activity of a mixture of 13-cis and 13'-cis isomers was significantly higher compared to the all-trans or 9'-cis isomeric forms (Fig. 4). Complete suppression of growth by 13- or 13'-cis isomer was achieved at 20 µM after 48 h of incubation, however, significant suppression was found in 13'-cis isomer even after 24 h of incubation (Fig. 5). These results suggest that 13'-cis fucoxanthin has greatest inhibitory effect on the growth of HL-60 cells, followed by 13-cis isomer and all-trans or 9'-cis isomers.

The present study confirmed that all-trans fucoxanthin significantly inhibits the cell growth in Caco-2, PC-3 and LNCap cells (Fig. 6 and 7). In addition, the results in Fig. 6
and 7 also demonstrate the important finding that 13-cis or 13’-cis fucoxanthin show a higher inhibitory effect on the growth of above cancer cell lines as compared to their all-trans form. Apoptosis induction has been reported to be the biochemical mechanism by which fucoxanthin exerts an inhibitory effect on cancer cells (Hosokawa et al., 1999; Kotake-Nara et al., 2005; Hosokawa et al., 2004; Kotake-Nara et al., 2001). Although the low concentration, a mixture of 13-cis and 13’-cis fucoxanthin (10 µM) induced a significant and dose-dependent increase in cellular DNA fragmentation in HL-60 cells (Fig. 8 (A)). A significant difference was also found in the Caco-2 cells treated with a mixture of 13-cis and 13’-cis isomers (10 µM) for 24 h (Fig. 8 (B)). However, differences in level of fucoxanthin that induces apoptosis between the present study and other previous studies could mainly be due to varied experimental conditions.

In an attempt to explore the effects of fucoxanthin on apoptosis regulating proteins, we examined the expression of Bel-2, which suppress programmed cell death. A remarkable difference between all-trans fucoxanthin and a mixture of 13-cis and 13’-cis fucoxanthin in terms of apoptosis induction was observed. It is possible that suppressing the expression of apoptosis down-regulating proteins could be one of the mechanism by fucoxanthin isomers induce apoptosis.

Due to the steric hindrance of cis configuration, it is possible that 13-cis and 13’-cis fucoxanthin isomerize to trans form in the cellular medium (Fig. 11). The bending structure of cis isomers (Fig. 1) might also contribute to their reduced incorporation into the cells (Fig. 10 (C) and (D)). On the other hand, the level of incorporated cis isomers remaine without a decrease during 48 h of incubation (Fig. 11 (C) and (D)), while all-trans fucoxanthin decreased after 12-24 h of incubation (Fig. 11 (A)-(D)).

The present study demonstrates the stronger inhibitory effect of 13-cis and 13’-cis fucoxanthin on the growth of cancer cells as compared to their all-trans counterparts, although the incorporation rate of cis isomers into the cell was slower than all-trans.

It has been suggested that carotenoids themselves or their derivatives of carotenoids modulate the expression of many proteins participating in several pathways related to cancer cell proliferation (Sharoni et al., 2003). Thus, the stronger effect exhibited by the cis isomers can be attributed to the differences in the modulation activity of each fucoxanthin isomer. In the initial expression of multiple proteins, the initial effect of carotenoids or carotenoid derivatives will involve the direct interaction of carotenoid molecules or carotenoid derivatives with transcriptional factors, e.g. with ligand-activated nuclear receptors or indirect modification of transcriptional activity. Interaction of carotenoid or carotenoid derivative as a ligand on a receptor is strongly affected by the stereochemical conformation of both molecules. Ligand activity of
carotenoids or caroptenoid derivatives is dependent on their stereochemical affinity and specificity to each receptor. It is therefore possible that the stronger antiproliferative effect of 13-*cis* and 13’-*cis* fucoxanthin on cancer cells, as evidenced in this study would be due to the stereo-chemically favorable structure of these *cis* isomers on the modulation of transcriptional factors responsible for cancer cell proliferation. However, well structured studies are required to decipher the exact mechanisms.

5. Conclusion

From the present study it can be concluded that anti-proliferative effect of fucoxanthin is dependent on its isomeric structure. *Cis* forms of fucoxanthin were found to exert higher inhibitory effect compared to their trans counterparts. Further, it is possible that the stronger anti-proliferative and inhibitory effect of *cis* isomers of fucoxanthin could be due to the steric hindrances offered by their structure. However, it should be mentioned that uptake and incorporation of all-*trans* form of fucoxanthin into cellular lipids is higher compared to its *cis* counterparts. Finally, it is possible that down-regulation of apoptosis reducing proteins could be one of the mechanisms by which *cis*-fucoxanthin isomers exert higher inhibitory effect. It is thus suggested that further studies are needed to clearly decipher the exact mechanisms as to how stereo isomers of fucoxanthin exhibit different level of inhibitory effect against cancer cells.

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References


Figure Legends

Fig. 1. Structure of all-trans and cis fucoxanthin.

Fig. 2. Reversed phase HPLC of fucoxanthin isomers obtained from algal lipids by silica gel chromatography (A) and changes in the isomeric mixtures after incubation at 5°C for 2 month (B).

Fig. 3. Separation of 13- and 13’-cis fucoxanthin isomers by normal phase HPLC.

Fig. 4. Comparison of viability of HL-60 cells incubated with all-trans fucoxanthin, 9’-cis fucoxanthin, and a mixture of 13-cis and 13’-cis fucoxanthin. HL-60 cells were incubated with 20 µM fucoxanthin isomers for 48 h. Cell viability was measured by trypan blue dye exclusion. Values are means±SD, n=3. The asterisk indicates a value significantly different from the control value (P<0.01). aSignificant difference from all-trans fucoxanthin (P<0.05).

Fig. 5. Comparison of viability of HL-60 cells incubated with all-trans fucoxanthin, 13-cis fucoxanthin, and 13’-cis fucoxanthin. HL-60 cells were incubated with 20 µM fucoxanthin isomers for 24h and 48 h. Cell viability was measured by trypan blue dye exclusion. Data represent cell viability expressed as a percentage of the control, which was taken to 100%. Values are means±SD, n=3. The asterisk indicates a value significantly different from the control value (P<0.01). a,bSignificant difference from all-trans fucoxanthin (a:P<0.05; b:P<0.01).

Fig. 6. Cell viability of Caco-2 cells (A), PC-3 cells (B), and LNCap cells (C) treated with all-trans fucoxanthin (open square) and a mixture of 13-cis and 13’-cis fucoxanthin (open circle). Cells were incubated in culture medium with 10 µM fucoxanthin isomers. Viability was measured by WST-1 assay. Data represent cell viability expressed as a percentage of the control, which was taken to 100%. Values are means±SD, n=3. The asterisk indicates a value significantly different from the control value (P<0.01). aSignificant difference from all-trans fucoxanthin (P<0.01).

Fig. 7. Cell viability of Caco-2 cells treated with all-trans fucoxanthin (solid diamond), 13-cis fucoxanthin (solid square), and 13’-cis fucoxanthin (solid triangle). Cells were incubated in culture medium with 10 µM and 20 µM fucoxanthin isomers. Viability was measured by WST-1 assay. Values are means±SD, n=3. The asterisk indicates a value significantly different from the control value (P<0.01). aSignificant difference from all-trans fucoxanthin (P<0.01). bSignificant difference from 13-cis fucoxanthin (P<0.01).
Fig. 8. DNA fragmentation in HL-60 (A) and Caco-2 (B) cells treated with all-trans fucoxanthin and a mixture of 13-cis and 13’-cis fucoxanthin. Cells were incubated in culture medium with 10 µM fucoxanthin isomers. DNA fragmentation was measured by a sandwich enzyme immunoassay using anti-histone antibody and anti-DNA antibody. Values are means±SD, n=3. In each incubation time, the asterisk indicates a value significantly different from the control value (P<0.01). aSignificant difference from all-trans fucoxanthin (P<0.01). a,bSignificant difference from all-trans fucoxanthin (a: P<0.05; b: P<0.01).

Fig. 9. Expression of Bcl-2 protein in HL-60 cells treated with all-trans fucoxanthin and a mixture of 13-cis and 13’-cis fucoxanthin. HL-60 cells were incubated in cultured medium containing 10 µM and 20 µM fucoxanthin isomers for 24 h or 48 h. Cellular protein was extracted, and levels of Bcl-2 protein were detected using Western blot analysis.

Fig. 10. Changes in the concentration of fucoxanthin isomer in the cellular lipids treated HL-60 cells with all-trans fucoxanthin and a mixture of 13-cis and 13’-cis fucoxanthin. HL-60 cells were incubated in cultured medium containing 2.5 µM all-trans fucoxanthin (A), 5.0 µM all-trans fucoxanthin (B), 2.5 µM a mixture of 13-cis and 13’-cis fucoxanthin (C), or 5.0 µM a mixture of 13-cis and 13’-cis fucoxanthin (D). Cellular lipids were extracted with chloroform/methanol (2:1, v/v) and each fucoxanthin isomer was evaluated quantitatively by HPLC. All-trans fucoxanthin concentration was indicated as solid square (A and B) or solid circle (C and D), while a mixture of 13-cis and 13’-cis fucoxanthin mixture as open square (A and B) or open circle (C and D).

Fig. 11. Changes in the concentration of fucoxanthin isomer in the RPML1640 medium after addition of all-trans fucoxanthin and a mixture of 13-cis and 13’-cis fucoxanthin. The cultured medium containing 10 µM all-trans fucoxanthin (A) and 10 µM a mixture of 13-cis and 13’-cis fucoxanthin (B) was incubated for 48 h. Fucoxanthin was extracted with chloroform/methanol (2:1, v/v) and each fucoxanthin isomer was evaluated quantitatively by HPLC. All-trans fucoxanthin concentration was indicated as solid square (A) or solid triangle (B), while a mixture of 13-cis and 13’-cis fucoxanthin mixture as solid circle (A) or solid diamond (B).
all-trans Fucoxanthin

9'-cis Fucoxanthin

13'-cis Fucoxanthin

13- cis Fucoxanthin

Fig. 1
Absorbance at 450 nm

Retention time (min)

(A) All \textit{trans}-fucoxanthin

(B) All \textit{trans}-fucoxanthin

13- and 13'\textit{cis} Fucoxanthin

9'-\textit{cis} Fucoxanthin

13- and 13'-\textit{cis} Fucoxanthin

Fig. 2
Fig. 3

Absorbance at 450 nm

Retention time (min)

13'-cis Fucoxanthin

13-cis Fucoxanthin
Fig. 4

Viable cell number (1X10^4 cells/mL)

- Control
- All-trans
- 9'-Cis
- 13-Cis+13'-cis

* Significant difference
Fig. 5

Viable cell number (1x10^4 cells/mL)

24h

Control  All-trans  13-Cis  13'-Cis

48h

Control  All-trans  13-Cis  13'-Cis
**Fig. 6**

- **(A)**
  - Incubation time (h) vs. Viability (%)
  - Symbols and markers indicate significant changes over time.
  - Asterisks denote statistical significance.

- **(B)**
  - Similar plot with additional markers indicating specific time points.

- **(C)**
  - Additional plot with similar data representation.
Fig. 7

Incubation time (h) vs. Viability (%)

- 10μM
- 20μM

* indicates significant difference from control.

(a) and (b) indicate different groups.

Graph shows the decline in viability over time for both concentrations.
Fig. 9

**Bcl-2 (26kDa)**

**β-Actin (43kDa)**

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Fig. 9
Fig. 10
Fig. 11

(A) Relative % vs. Incubation time (h)

(B) Relative % vs. Incubation time (h)