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 (*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 apoptotis inducing activity.

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 lycopne 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 lycopne 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 ^{o}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^{o}C for 2 months in the dark to isomerize with minimum docomposition. 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 $CDCl_3$. The 1D-NMR (^{1}H) and 2D-NMR (^{1}H-^{1}H COSY, ^{1}H-^{1}H 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% CO_2 at 37 ^{o}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. Mesurement 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 ($1.5x10^6$ 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 Na_3VO_4. Further, cell lysates were centrifuged at 4^oC, 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 lpids 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 $\pm$ 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^oC 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 ^{1}H-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 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 geometreical 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 Bc1-2 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-denpendent 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 Bcl-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* configulation, 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 carotenopid 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 moudulation 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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Figure lgends

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^{o}C for 2 month (B).

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

Fig. 4. Comparision 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). [a]Significant difference from all-trans fucoxanthin (P<0.05).

Fig. 5. Comparision 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,b]Significant 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). [a]Significant difference from all-*trans* fucoxanthin (P<0.01).

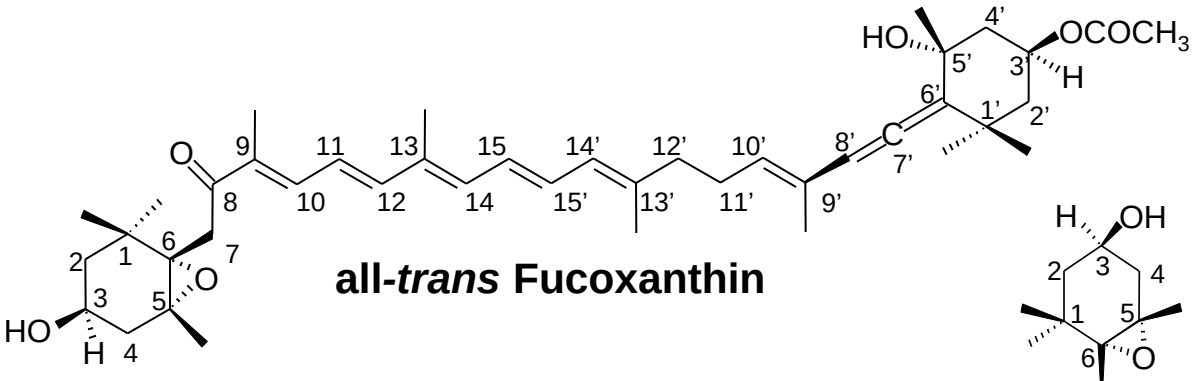
Fig. 7. Cell viability of Caco-2 cells treated with all-*trans* fucoxanthin (solid diamond), 13-*cis* fucoxanthin (solid square), and 13'-*cis* fucoxanthin (solid triangle). Cells were incubated in culture medium with 10 μM and 20 μM fucoxanthin isomers. Viability was measured by WST-1 assay. Values are means±SD, n=3. The asterisk indicates a value significantly different from the control value (P<0.01). [a]Significant difference from all-*trans* fucoxanthin (P<0.01). [b]Significant 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 ($P<0.01$). [a]Significant difference from all-*trans* fucoxanthin ($P<0.01$). [a,b]Significant difference from all-trans fucoxanthin (a:$P<0.05$; b:$P<0.01$).

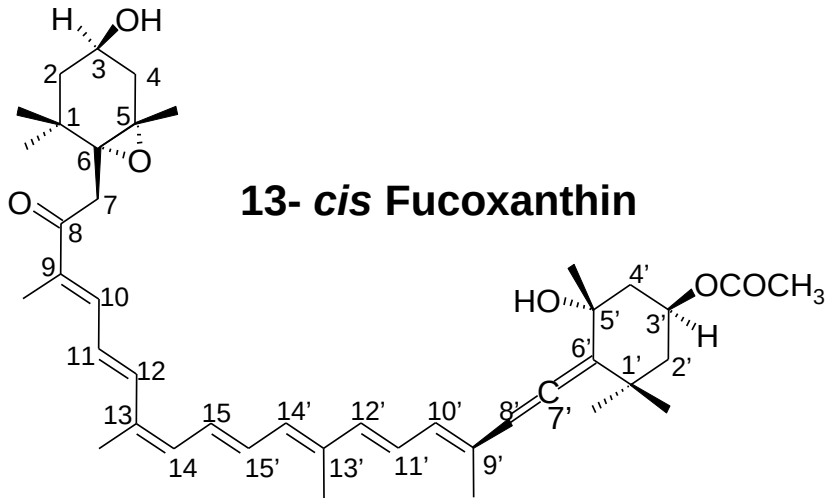
Fig. 9. Expression of Bcl-2 protein in HL-60 cells treated with all-*trans* fucoxanthin and a mixture of 13-*cis* and 13'-*cis* fucoxanthin. HL-60 cells were incubated in cultured medium containing 10 μM and 20 μM fucoxanthin isomers for 24 h or 48 h. Cellular protein was extracted, and levels of Bcl-2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

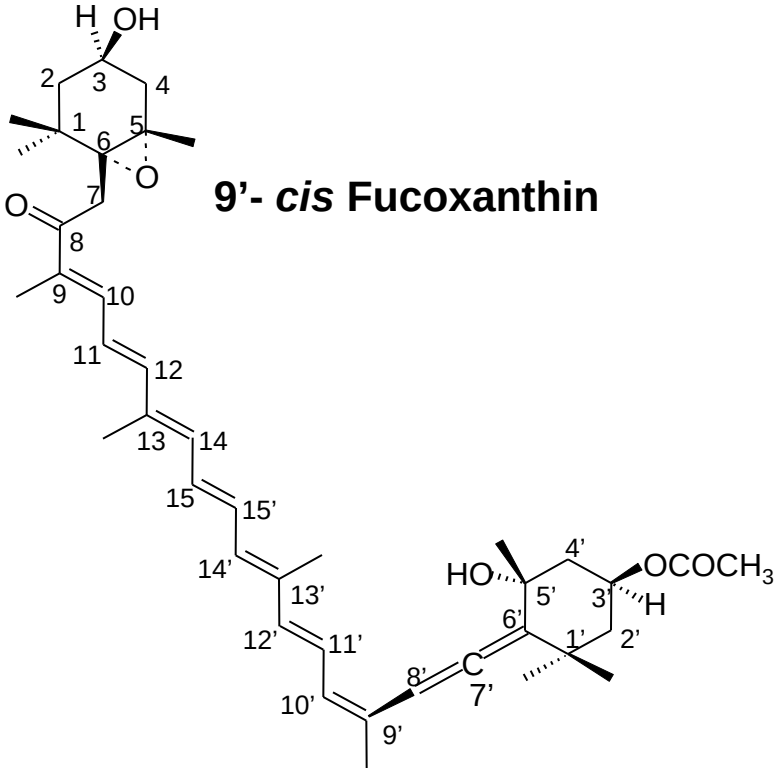
Fig. 11. Changes in the concentration of fucoxanthin isomer in the RPML1640 medium after addition of all-*trans* fucoxanthin and a mixture of 13-*cis* and 13'-*cis* fucoxanthin. The cultured medium containing 10 μM all-*trans* fucoxanthin (A) and 10 μM a mixture of 13-*cis* and 13'-*cis* fucoxanthin (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).



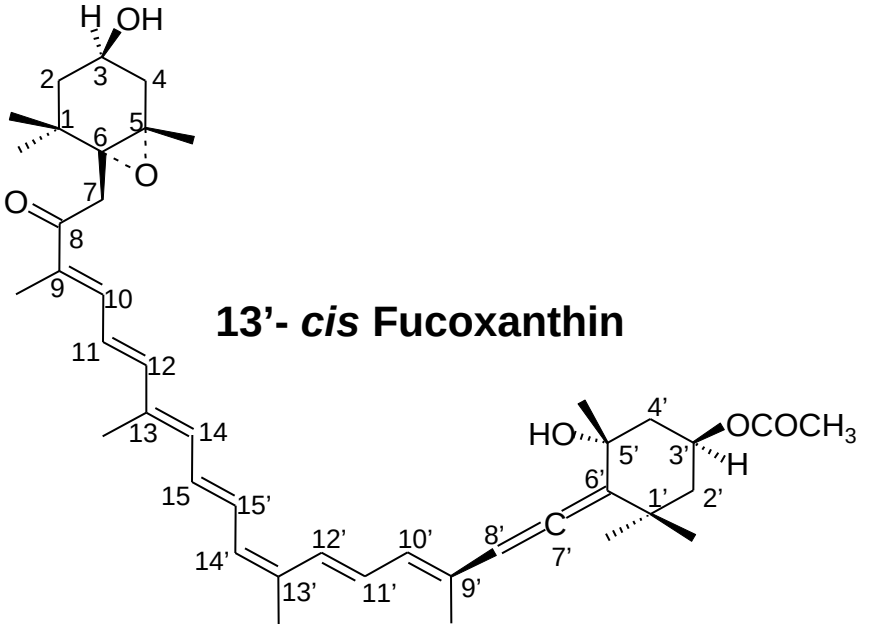
all-*trans* Fucoxanthin



13- *cis* Fucoxanthin



9'- *cis* Fucoxanthin



13'- *cis* Fucoxanthin

Fig. 1

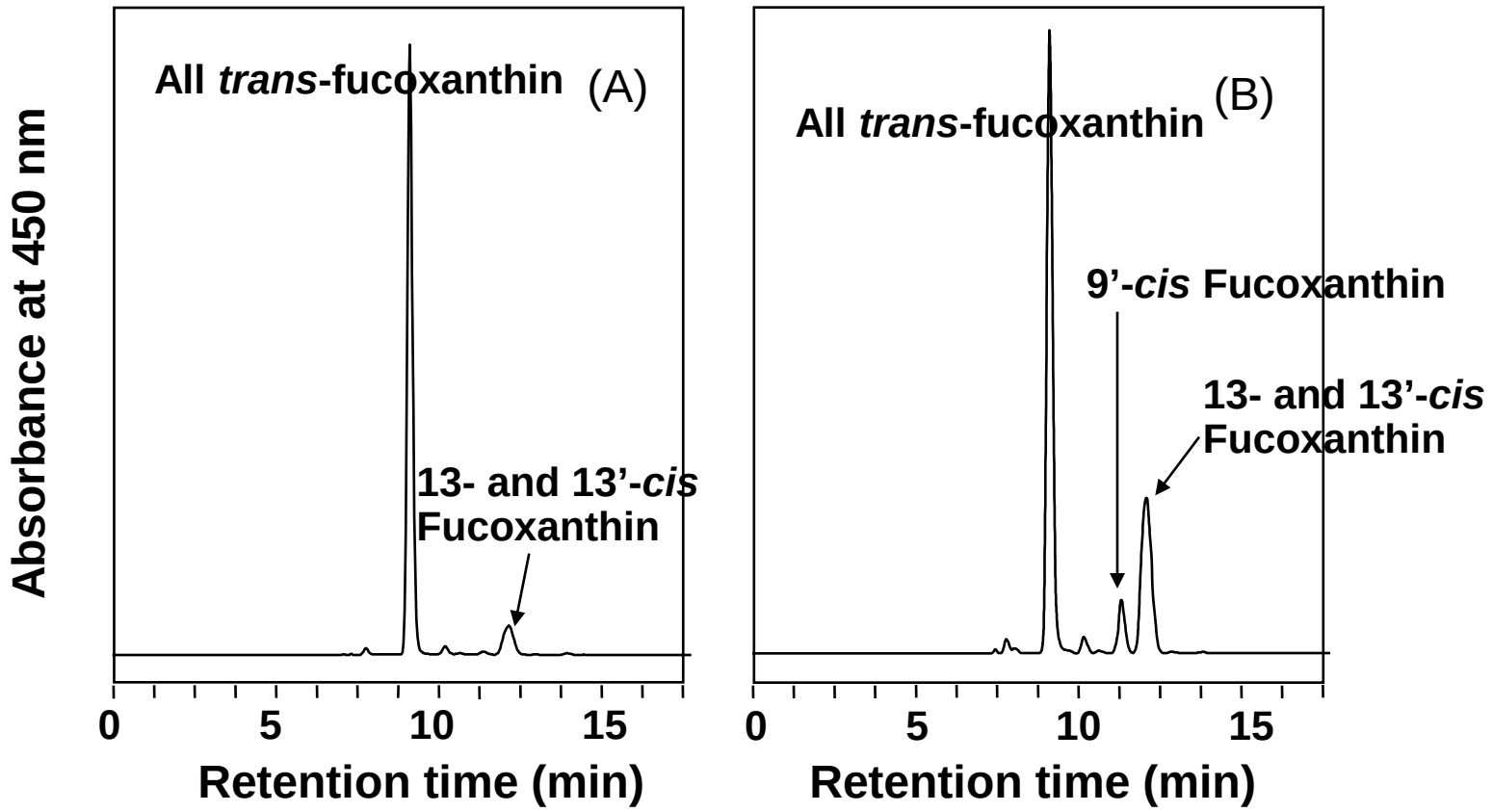

All *trans*-fucoxanthin
(A)
13- and 13'-*cis*
Fucoxanthin
Absorbance at 450 nm
0
5
10
15
Retention time (min)
All *trans*-fucoxanthin
(B)
9'-*cis* Fucoxanthin
13- and 13'-*cis*
Fucoxanthin
0
5
10
15
Retention time (min)

Fig. 2

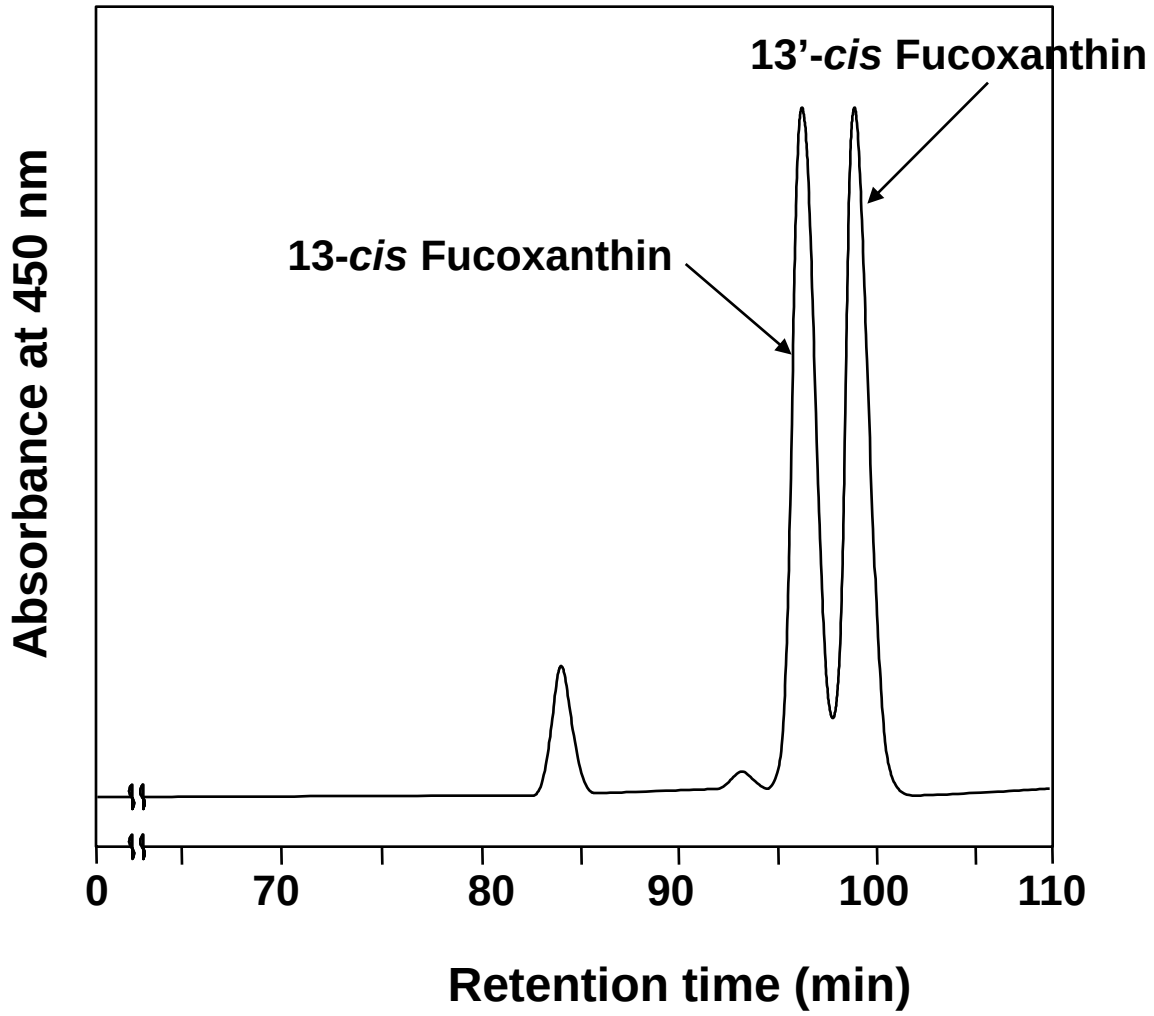

13'-*cis* Fucoxanthin
13-*cis* Fucoxanthin
Absorbance at 450 nm
0
70
80
90
100
110
Retention time (min)

Fig. 3

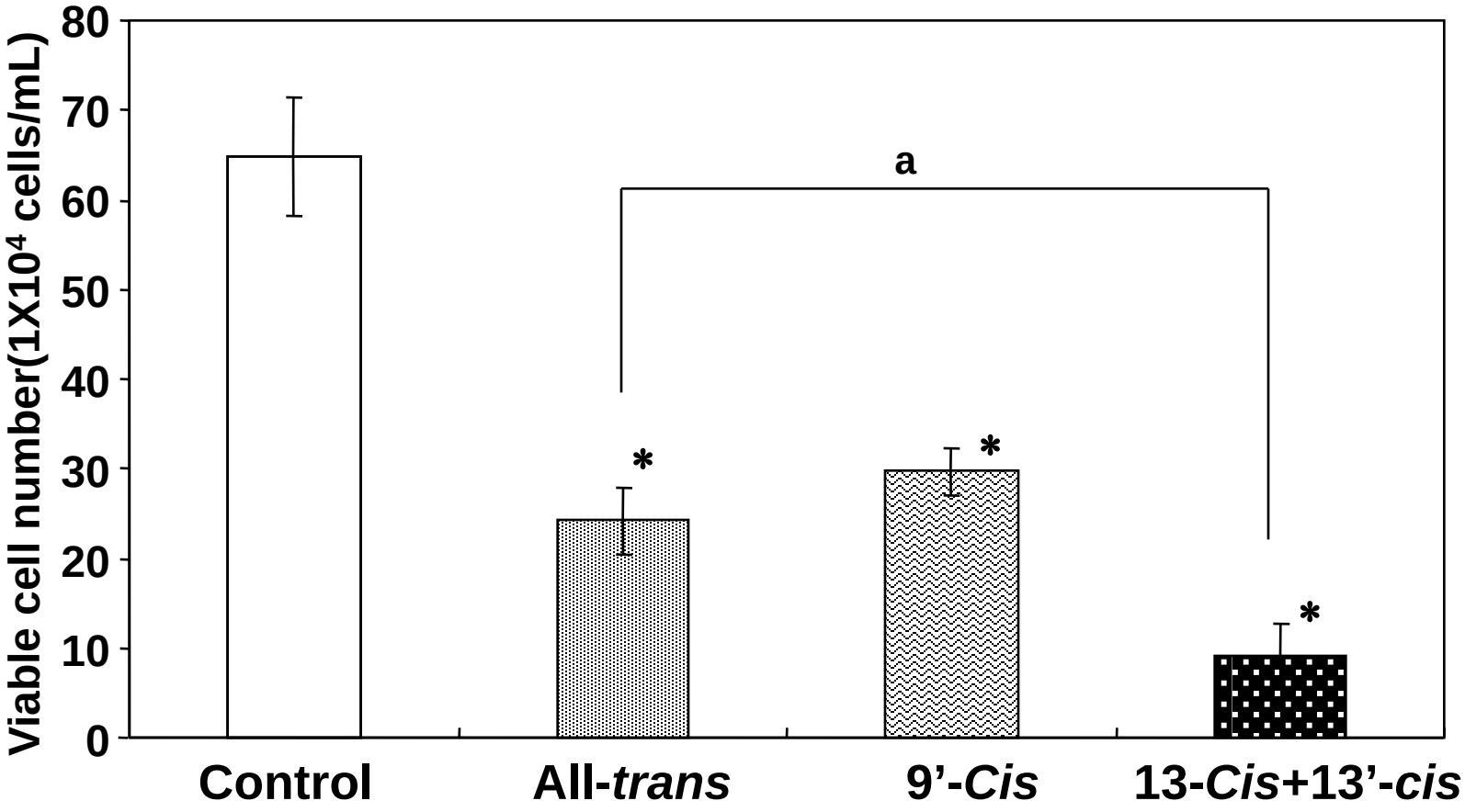

Viable cell number(1X10⁴ cells/mL)
80
70
60
50
40
30
20
10
0
a
*
*
*
Control
All-trans
9'-Cis
13-Cis+13'-cis

Fig. 4

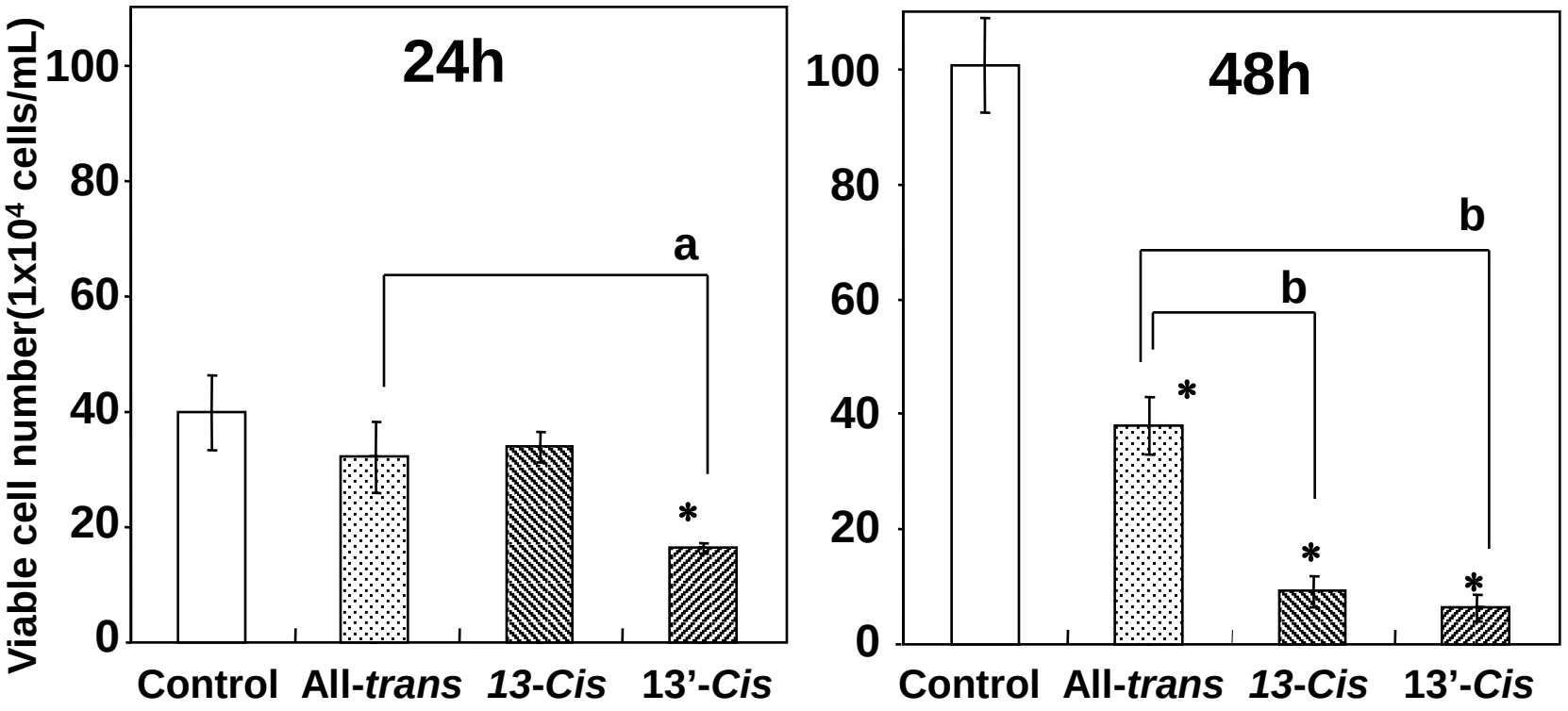

24h
48h
Viable cell number(1x10⁴ cells/mL)
100
80
60
40
20
0
a
b
b
*
*
*
*
Control
All-trans
13-Cis
13'-Cis
Control
All-trans
13-Cis
13'-Cis

Fig. 5

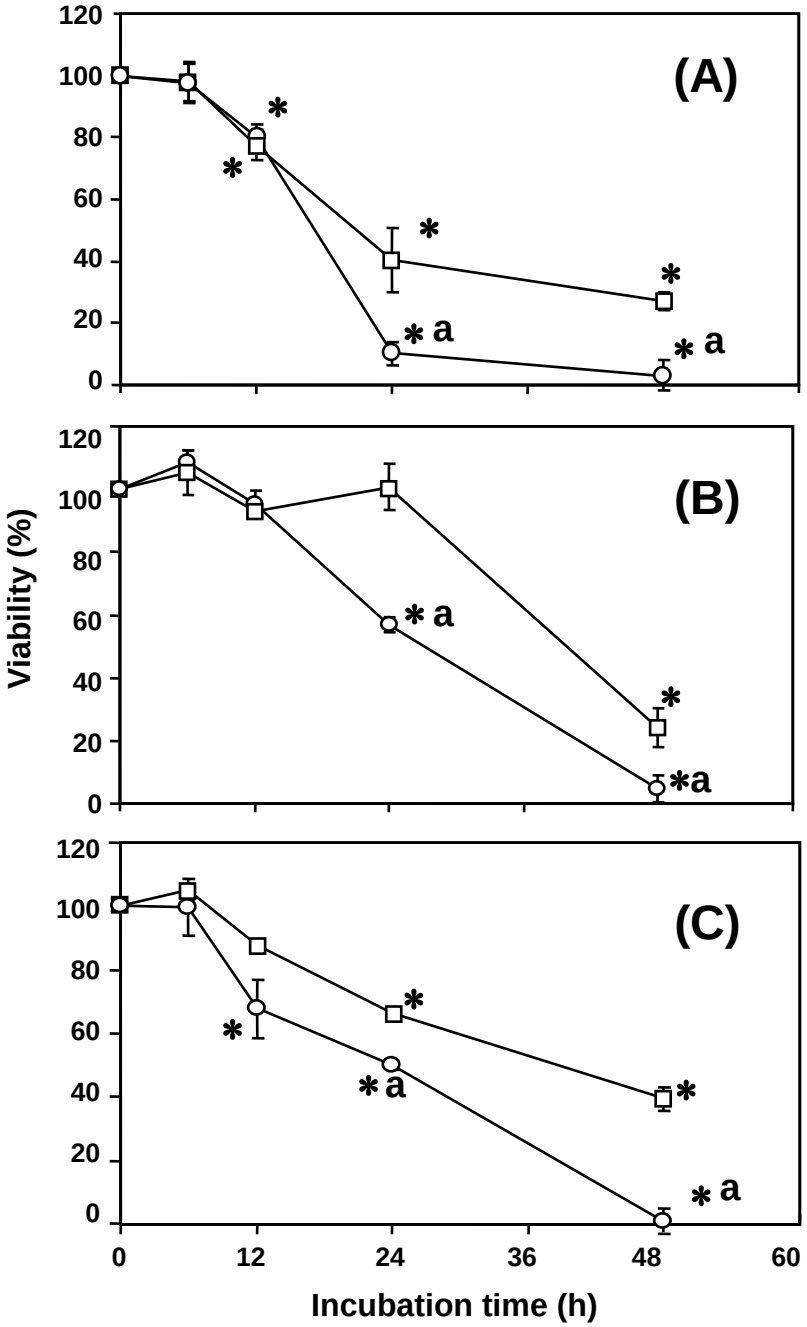

(A)
(B)
(C)
Viability (%)
Incubation time (h)
0
20
40
60
80
100
120
0
12
24
36
48
60
a

Fig. 6

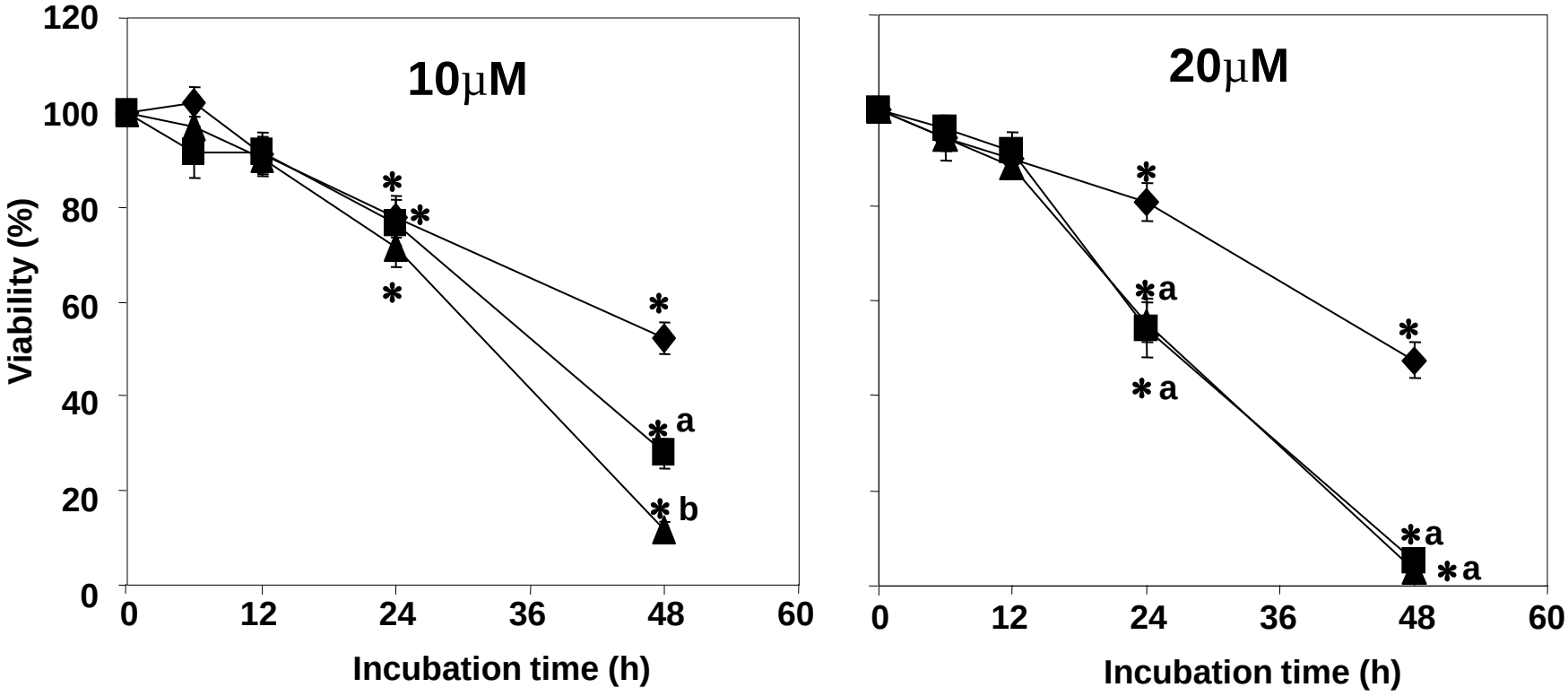

10μM
20μM
Viability (%)
Incubation time (h)
Incubation time (h)
0
20
40
60
80
100
120
0
12
24
36
48
60
a
b
a
a
a

Fig. 7

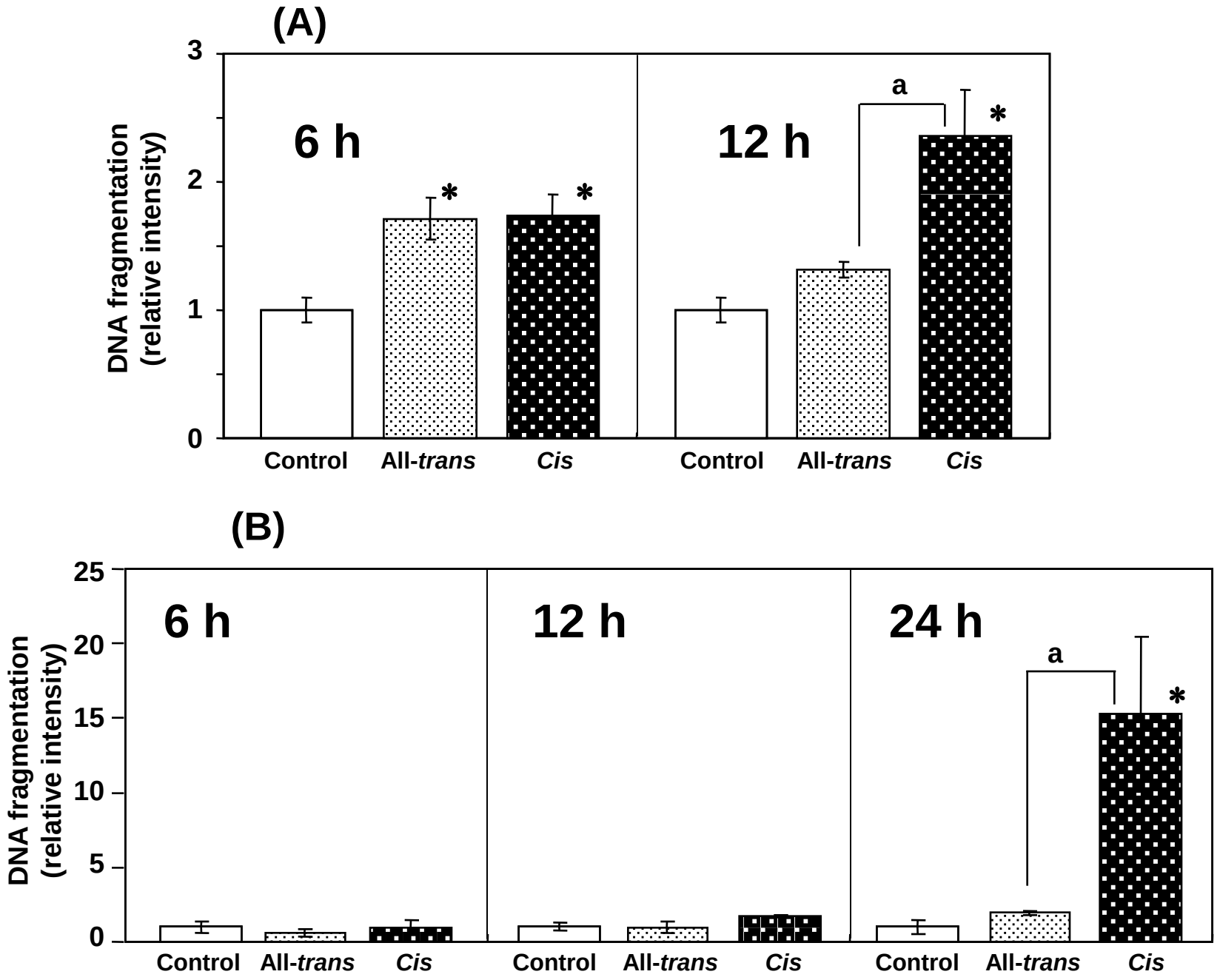

(A)
DNA fragmentation
(relative intensity)
3
2
1
0
6 h
12 h
a
*
*
*
Control
All-trans
Cis
Control
All-trans
Cis
(B)
DNA fragmentation
(relative intensity)
25
20
15
10
5
0
6 h
12 h
24 h
a
*
Control
All-trans
Cis
Control
All-trans
Cis
Control
All-trans
Cis

Fig. 8

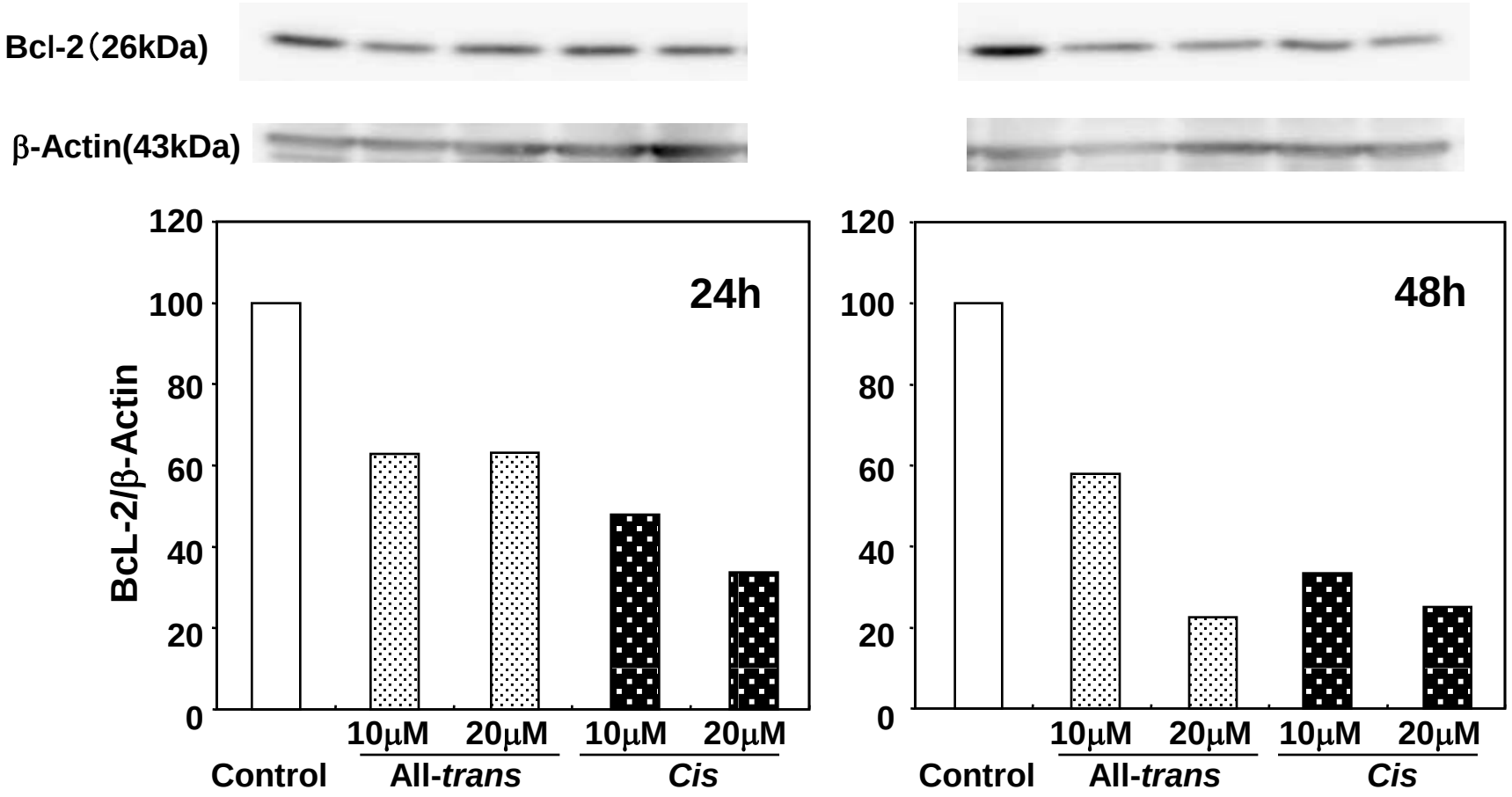

Bcl-2（26kDa)
β-Actin(43kDa)
BcL-2/β-Actin
120
100
80
60
40
20
0
24h
48h
10μM
20μM
10μM
20μM
Control
All-trans
Cis

Fig. 9

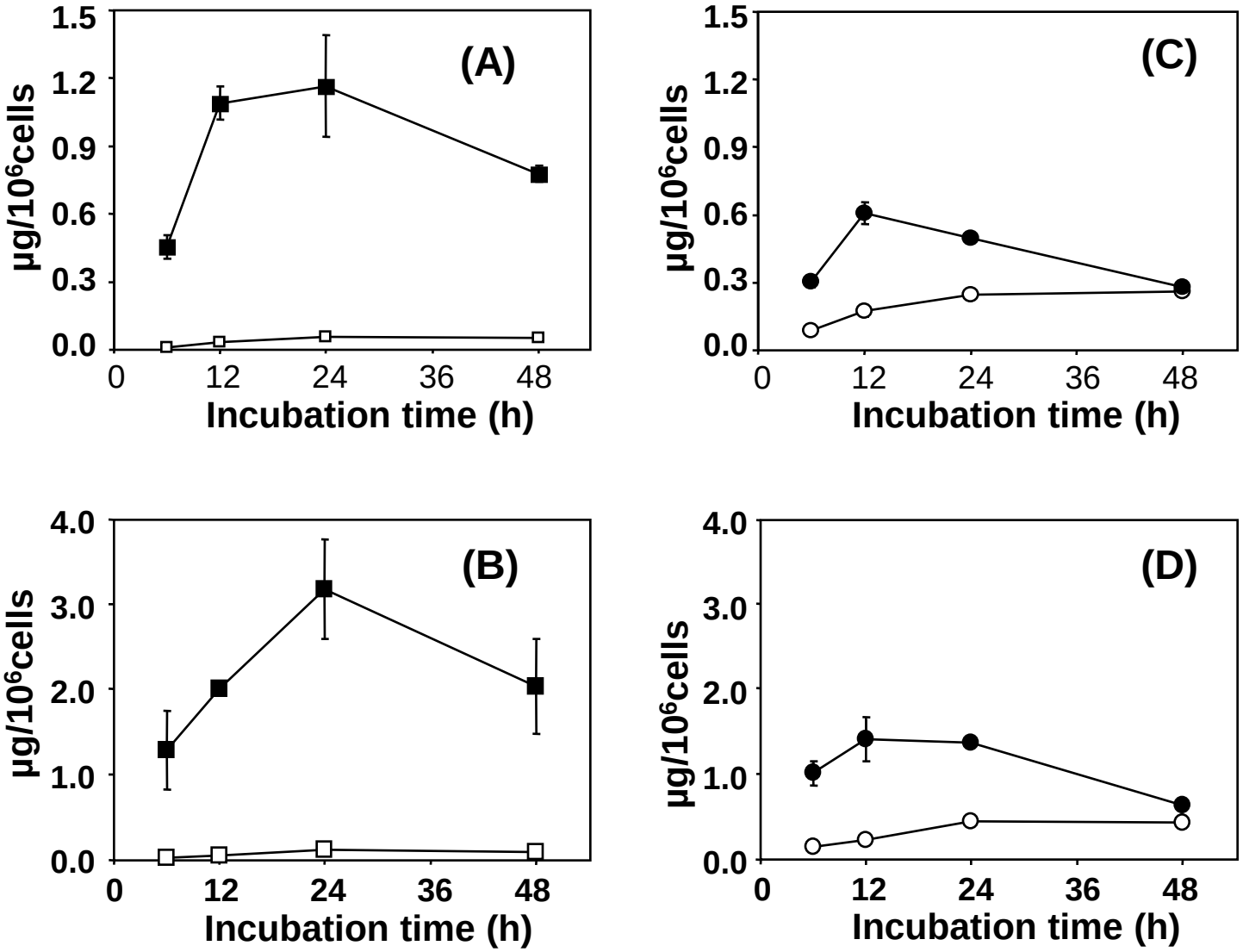

(A)
(B)
(C)
(D)
μg/10^6cells
Incubation time (h)
0
12
24
36
48
0.0
0.3
0.6
0.9
1.2
1.5
1.0
2.0
3.0
4.0

Fig. 10

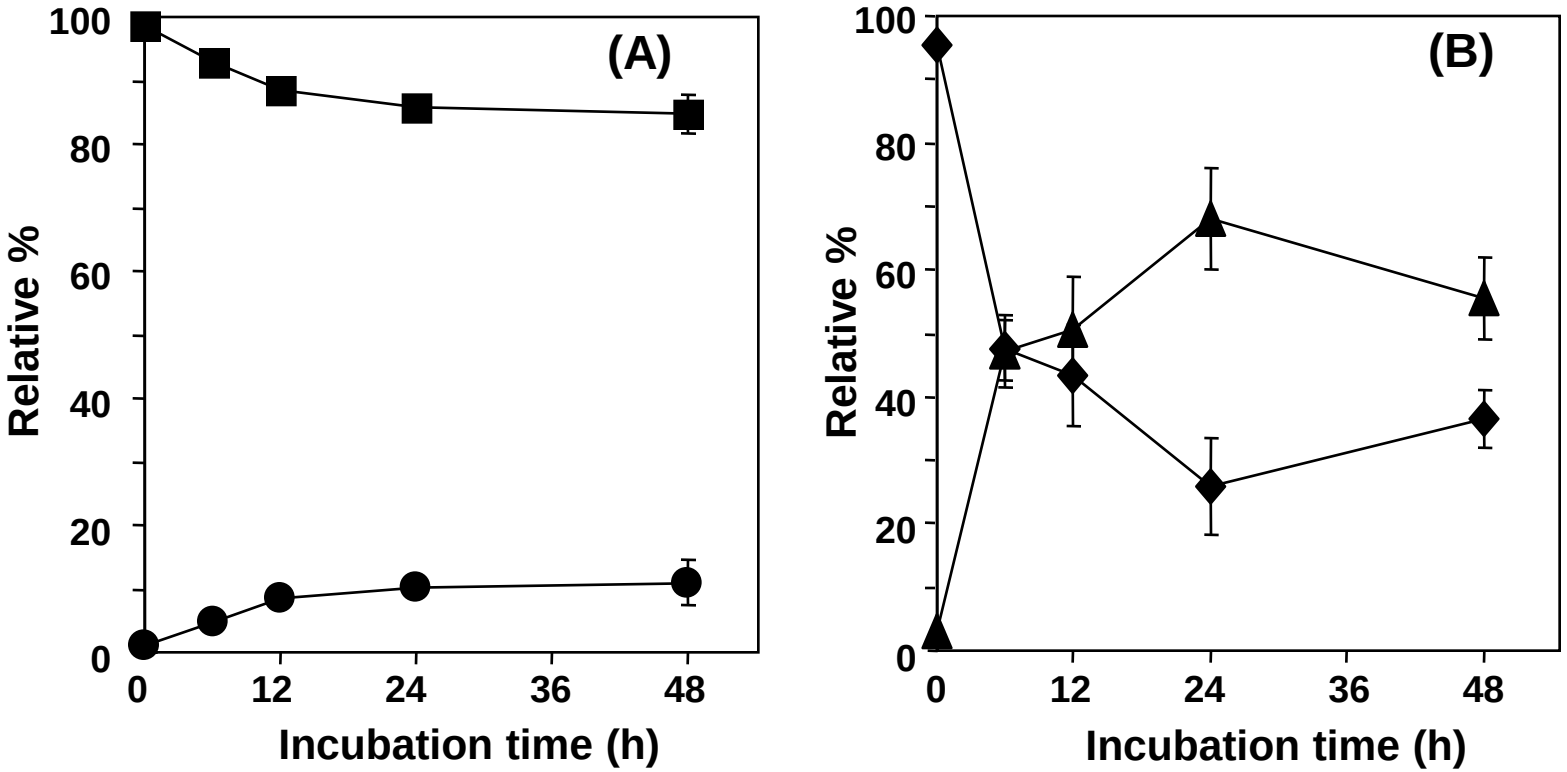

(A)
Relative %
100
80
60
40
20
0
0
12
24
36
48
Incubation time (h)
(B)
Relative %
100
80
60
40
20
0
0
12
24
36
48
Incubation time (h)

Fig. 11