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European Journal of Pharmacology 360 (1998) 65–71

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Protective actions of 5′-*n*-alkylated curcumins on living cells suffering from oxidative stress

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Received 6 April 1998; revised 20 August 1998; accepted 25 August 1998

Abstract

To elucidate the roles of 5′-*n*-alkyl chains of curcumin derivatives in their protective actions on cells against oxidative stress, we compared the efficacy of curcumin and 5′-alkylated curcumins to inhibit the peroxidation of linoleic acid and to protect rat thymocytes suffering from H_2O_2-induced oxidative stress with their permeation into cells. The inhibitory action of 5′-*n*-alkylated curcumins on lipid peroxidation increased as the length of hydrocarbon chains of 5′-*n*-alkylated curcumins was prolonged. This potency order was not confirmed for the protective actions of 5′-*n*-alkylated curcumins on cells against oxidative stress. Among 5′-alkylated curcumins, the most potent protective action was observed for 5′-*n*-C_3H_7-curcumin because it had the greatest permeation into the cells. Further increases in the length of hydrocarbon chains (up to *n*-$C_{19}H_{39}$) of 5′-*n*-alkylated curcumins greatly attenuated their protective actions by reducing their permeation into the cells.

Keywords: Curcumin; Antioxidant activity; Hydrogen peroxide; Alkylated curcumin

1. Introduction

Zingiber cassumunar is a medical ginger and is also used as a spice like turmeric. Cassumunins A and B, complex curcuminoids extracted from the rhizomes of *Z. cassumunar*, exert inhibitory actions on lipid peroxidation (Masuda et al., 1993; Masuda and Jitoe, 1993) and protective actions on living cells suffering from oxidative stress (Nagano et al., 1997; Kanemaru et al., 1998). The chemical structures of cassumunins A and B were determined (Masuda et al., 1993) and subsequently confirmed by synthesis (Masuda et al., 1998). Their chemical structures include that of curcumin which is widely distributed in the ginger rhizomes and is known to have antioxidant activity (Sarma, 1976; Toda et al., 1985). Cassumunins A and B are 5′-substituted derivatives of curcumin (Fig. 1). However, the potencies of cassumunins A and B for protection of cells suffering from the oxidative stress induced by hydrogen peroxide (H_2O_2) are greater than that of curcumin (Nagano et al., 1997; Kanemaru et al., 1998). Therefore, to find the roles of 5′-substitutes of curcumin derivatives in the modification of antioxidant activities, we examined the actions of 5′-*n*-alkylated curcumins on living cells suffering from oxidative stress induced by H_2O_2. For the purpose, we used a flow cytometer with ethidium bromide, a fluorescent dye for identifying dead cells or cells having compromised membranes.

2. Materials and methods

2.1. Assay of inhibitory action on lipid peroxidation

The chemical assay was carried out according to a method described previously (Masuda et al., 1993). In brief, a micelle solution of 2 mM linoleic acid (Nacalai, Kyoto, Japan) was prepared by sonication for 1 min in 0.1 M sodium dodecyl sulfate–0.05 M phosphate buffer (pH 7.4). The solution (3 ml) was placed in a UV cell and 10 μl of 5 mM test sample in CH_3CN was added. After

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0014-2999/98/$ - see front matter
PII: S0014-2999(98)00635-9

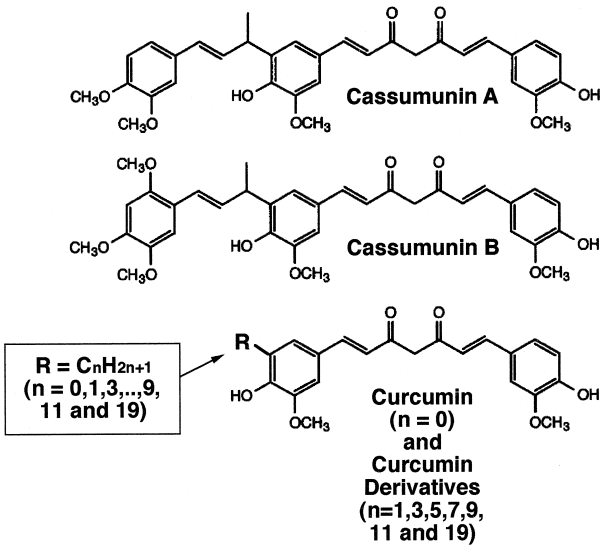

Fig. 1. Chemical structures of cassumunin A, cassumunin B, curcumin and 5′-alkylated curcumins.

thermal equilibration at 50°C for 15 min, 10 μl of 700 mM 2,2′-azo-bis-(2,4-dimethylvaleronitrile) solution (in CH_3CN) was added and stirred. Peroxidation of linoleic acid was monitored by recording the absorbance at 234 nm for 1 h with a Shimadzu UV-1200 spectrophotometer (Kyoto, Japan) equipped with a Taitec thermo controller SP-12 (Tokyo, Japan).

2.2. Assay of protective action on living cells suffering from oxidative stress

The assay was carried out according to methods described previously (Nagano et al., 1997; Kanemaru et al., 1998). Experiments were performed on thymocytes dissociated from thymus glands of 4-week-old Wistar rats (Nissin, Tokushima, Japan). Thymocytes were chosen for the experiments for the following reasons. First, these cells can be dissociated without treatment with proteolytic enzymes which could compromise the membranes of some cells during digestion. The viability of dissociated thymocytes was 93–97% under control condition. Second, thymocytes are suitable for flow cytometric analysis because of their spherical shape, size and homogeneity. The technique for dissociation of rat thymocytes was similar to that described previously (Chikahisa et al., 1996). In brief, thymus glands dissected from the rats were sliced at a thickness of 400–500 μm. Thereafter, the slices were gently triturated in chilled Tyrode's solution to dissociate single thymocytes. Tyrode's solution containing dissociated thymocytes was passed through a mesh (diameter, 53 μm) to remove residues.

To estimate the viability of thymocytes in the cell suspension, ethidium bromide (Molecular Probe, Eugene, USA) was used. Ethidium bromide was added to the cell suspension to achieve a final concentration of 10 μM. Ethidium, which is highly impermeant to intact membranes, cannot stain live cells while it can stain dead or

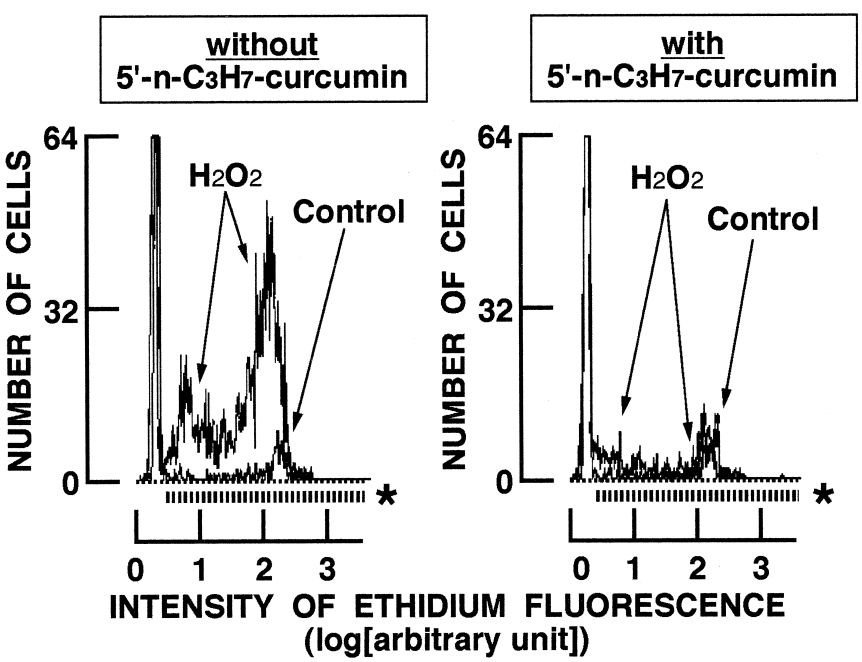

Fig. 2. Effect of 5′-*n*-C_3H_7-curcumin on the histogram of ethidium fluorescence obtained from 3000 thymocytes. The concentration of *n*-C_3H_7-curcumin was 1 μM. Cells were treated (right panel) or not treated with (left panel) the agent before the start of oxidative stress induced by 3 mM H_2O_2. The control histograms (as indicated by the arrow and Control) were superimposed on those obtained 3 h after the start of oxidative stress (as indicated with the arrows and H_2O_2). Dotted lines with asterisks under the histograms indicate cells showing ethidium fluorescence.

damaged cells because of the loss of membrane integrity (Haugland, 1997). Therefore, the measurement of ethidium fluorescence from the cells provides a clue to cell viability in the cell suspension. Fluorescence from cells incubated with ethidium bromide was measured 2–3 min after dye application because ethidium slightly stained some live cells during a prolonged exposure (longer than 30 min), and 2–3 min was enough time to stain dead and damaged cells. Ethidium fluorescence was measured with a flow cytometer (Cyto ACE-150, Japan Spectroscopic, Tokyo, Japan). The excitation wavelength for ethidium, 488 nm, was produced by an argon laser. Emission was detected at a wavelength of 600 ± 20 nm. The fluorescence histogram obtained from a programmed number of cells was analyzed by using JASCO VER.3XX software (Japan Spectroscopic) and a personal computer (PC-9801RX, NEC, Tokyo, Japan). Statistical analysis was performed with two-sample *t*-tests. A P value of < 0.05 was considered significant.

2.3. Chemicals

Curcumin was synthesized according to Pabon's method and was used after purification on Merck TLC silica gel plate 60 F254 (Art. No. 5744) developed with 5% CH_3OH in CH_2Cl_2. Cassumunins and 5′-*n*-alkylated curcumins were prepared as described previously (Masuda et al., 1997, 1998). The chemical structures of cassumunin A, cassumunin B, curcumin and 5′-alkylated curcumins are shown in Fig. 1. Other chemical reagents were purchased from Katayama Chemical Industries (Osaka, Japan) unless mentioned otherwise.

3. Results

3.1. Inhibitory actions of curcumin and 5-alkylated curcumins on peroxidation of linoleic acid

Curcumin exerted an inhibitory action on peroxidation of linoleic acid. The inhibition by 5′-alkylated curcumins of the peroxidation of linoleic acid increased as the length of 5′-*n*-alkyl chains of curcumin derivatives increased. The potencies of 5′-alkylated curcumins, relative to that of curcumin, to inhibit lipid peroxidation increased markedly up to 1.42, when the number of carbon atoms was increased from 0 to 5. Further increases in the number of carbons (up to 9) did not greatly augment the inhibitory action. The relative potency of 5′-*n*-C_9H_{19}-curcumin was 1.52.

3.2. Actions of curcumin and 5-alkylated curcumins on H_2O_2-induced increases in number of cells stained with ethidium

H_2O_2 at the concentration of 3 mM was lethal for thymocytes when the exposure lasted for 2 h or more (Okazaki et al., 1996; Nagano et al., 1997). Prolonged exposure to 3 mM H_2O_2 increased the number of cells stained with ethidium (Fig. 2), indicating a decrease in cell viability. The various 5′-alkylated curcumins were added into the cell suspension 1 h before the start of oxidative stress induced by 3 mM H_2O_2. Their protective actions against the oxidative stress of the cells were examined at 3 h after the start of oxidative stress. As shown in Fig. 3, pretreatment with 1 μM 5′-*n*-C_3H_7-curcumin attenuated the H_2O_2-induced increase in the number of cells stained

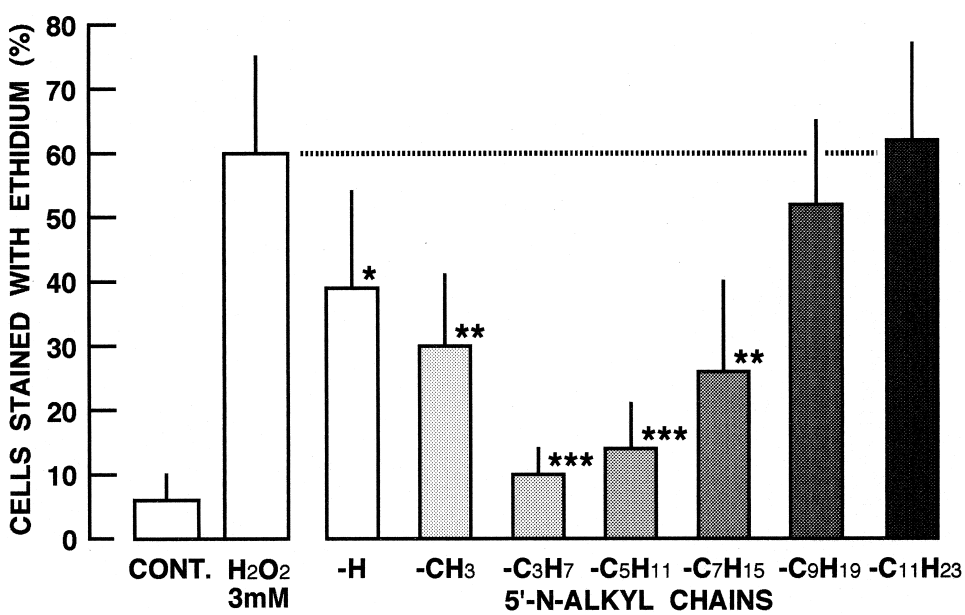

Fig. 3. Comparison of the effects of curcumin and 5′-alkylated curcumins (1 μM) on the H_2O_2-induced increases in the population of cells stained with ethidium. Each column and bar shows the average and S.D. of four experiments. Dotted line over the columns indicates the level of the H_2O_2-induced increase in the population of cells stained with ethidium. Asterisks indicate significant differences between H_2O_2-affected groups with and without treatment with the 5′-*n*-alkyl curcumins (∗: $P < 0.05$, ∗∗: $P < 0.01$, ∗∗∗: $P < 0.005$).

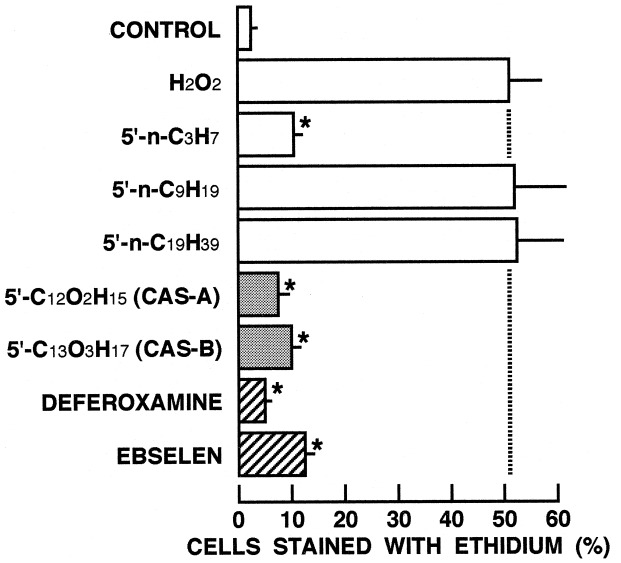

Fig. 4. Comparison of the effects of 5′-alkylated curcumins with those of compounds possessing antioxidant activities. Columns and bars show the averages and S.D. of four experiments. Dotted line indicates the level of H_2O_2-induced increase in the population of cells stained with ethidium. Asterisks indicate significant differences between H_2O_2-affected groups with and without treatments with cassumunin A or B (∗: $P < 0.005$).

with ethidium, indicating a protective action on the cells against the H_2O_2-induced oxidative stress. H_2O_2 at 3 mM increased the number of cells stained with ethidium in a time-dependent manner. This time-dependent increase was significantly suppressed in the presence of 1 μM 5′-*n*-C_3H_7-curcumin. Thus, after 3 h in the presence of 1 μM 5′-*n*-C_3H_7-curcumin the cells did not stain with ethidium whereas about 45% of the cell population was stained following a 3-h exposure to 3 mM H_2O_2.

Some of 5′-alkylated curcumins at 3 μM or more affected the measurement of ethidium fluorescence because of their own fluorescence. The protective actions of the agents on the cells suffering from H_2O_2-induced oxidative stress were thus compared at 1 μM. The various 5′-alkylated curcumins were applied to the cell suspension 1 h before the start of oxidative stress from the following reason. The intensities of fluorescence monitored from the cells treated with each 5′-alkylated curcumin increased time-dependently and reached steady state within 60 min after the start of their application. This result suggests that the cellular concentration of the various agents reached saturation within 60 min after the application. The effects of 5′-alkylated curcumins on the H_2O_2-induced increase in the number of cells stained with ethidium were examined at 3 h after the start of oxidative stress. As shown in Fig. 5, 3 mM H_2O_2 significantly increased the population of cells stained with ethidium while the curcumin derivatives with $-CH_3$, $-n$-C_3H_7, $-n$-C_5H_{11} and $-n$-C_7H_{15} in the 5′ position attenuated the H_2O_2-induced increase in the number of cells stained with ethidium (Fig. 3), indicating protective actions of these 5′-alkylated curcumins against the oxidative stress. As shown in Fig. 3, the protective effects of 5′-alkylated curcumins on the cells were not significant when the number of carbons in the 5′ position in substitutes of curcumin derivatives increased beyond 7. When 5′-*n*-C_3H_7-curcumin was given immediately after the start of oxidative stress induced by 3 mM H_2O_2, it was also effective to protect the cells, similarly to cassumunin A and cassumunin B (Nagano et al., 1997).

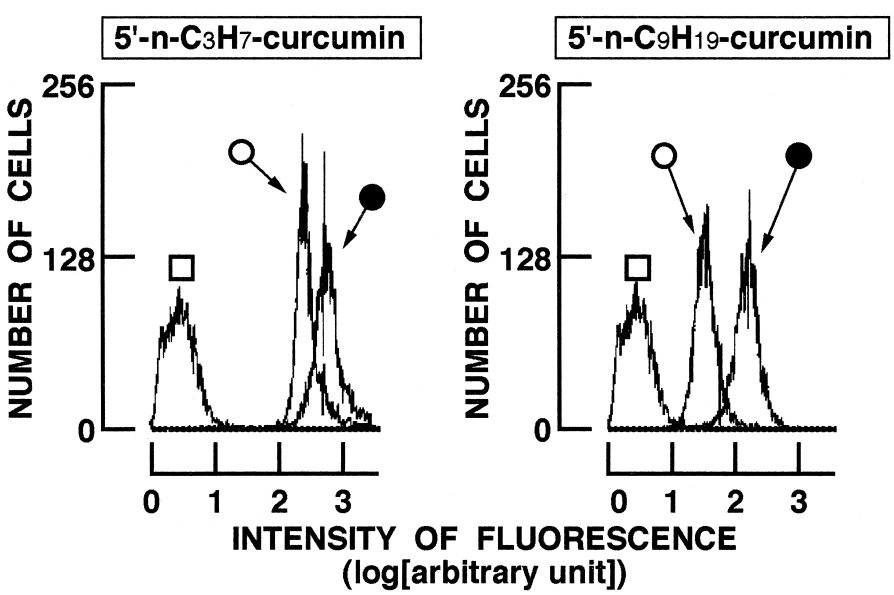

Fig. 5. Histograms of fluorescence obtained from the live (open circles) and dead (filled circles) cells treated with 3 μM 5′-*n*-alkyl curcumins (right panel: 5′-*n*-C_3H_7-curcumin; left panel: 5′-*n*-C_9H_{19}-curcumin). Open squares indicate the fluorescence histogram obtained from cells not treated with 5′-alkylcurcumins.

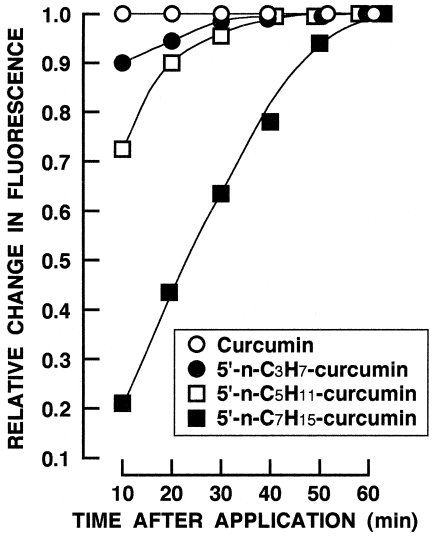

Fig. 6. Time-dependent changes in the intensity of fluorescence monitored from the cells treated with 5′-alkylcurcumins. Result shows an example from the average of three experiments.

3.3. Comparison of protective effect of 5′-n-C_3H_7-curcumin with protection by other chemical compounds

The effect of 1 μM 5′-*n*-C_3H_7-curcumin was compared, under the experimental conditions described above (Fig. 3), with the effects of 1 μM 5′-*n*-C_9H_{19}-curcumin, 1 μM 5′-*n*-$C_{19}H_{39}$-curcumin, 1 μM 5′-$C_{12}O_2H_{15}$-curcumin (cassumunin A), 1 μM 5′-$C_{13}O_3H_{17}$ (cassumunin B), 1 mM deferoxamine and 3 μM ebselen. As shown in Fig. 4, curcumin derivatives with 5′-*n*-C_9H_{19}– and 5′-*n*-$C_{19}H_{39}$– exerted no protective action on the cells suffering from H_2O_2-induced oxidative stress. This suggests the possibility that 5′-alkylated curcumins at 1 μM exert no protective action when the number of carbons in the 5′-*n*-alkyl chains is from 9 to 19. The efficacies of 5′-*n*-C_3H_7-curcumin and 5′-*n*-C_5H_{11}-curcumin to protect the cells against oxidative stress were almost similar to those of cassumunins A and B (Nagano et al., 1997) as shown in Fig. 4 although the chemical structures at 5′ position of cassumunins A and B (as curcumin derivatives) are more complex than those of the 5′-alkylated curcumins (Fig. 1). Therefore, –$C_{12}O_2H_{15}$ (cassumunin A) and –$C_{13}O_3H_{17}$ (cassumunin B) are not essential for the protective action against reactive oxygen species. Ebselen (3 μM) and deferoxamine (1 mM) as reference agents significantly reduced the H_2O_2-induced increase in the number of cells stained with ethidium (Fig. 4).

3.4. Difference in cellular concentration between 5′-alkylated curcumins

There was no linear structure–activity relationship for the protective action of 5′-alkylated curcumins on the cells against the H_2O_2-induced oxidative stress (Fig. 3) although the potency of 5′-alkylated curcumins to inhibit the peroxidation of linoleic acid increased as the length of the 5′-*n*-alkyl chain increased. To elucidate the mechanism involved in such a difference in potency order between the inhibitory action on lipid peroxidation and the protective action on the cells, we estimated the permeation of 5′-alkylated curcumins across the cell membranes into the cell,

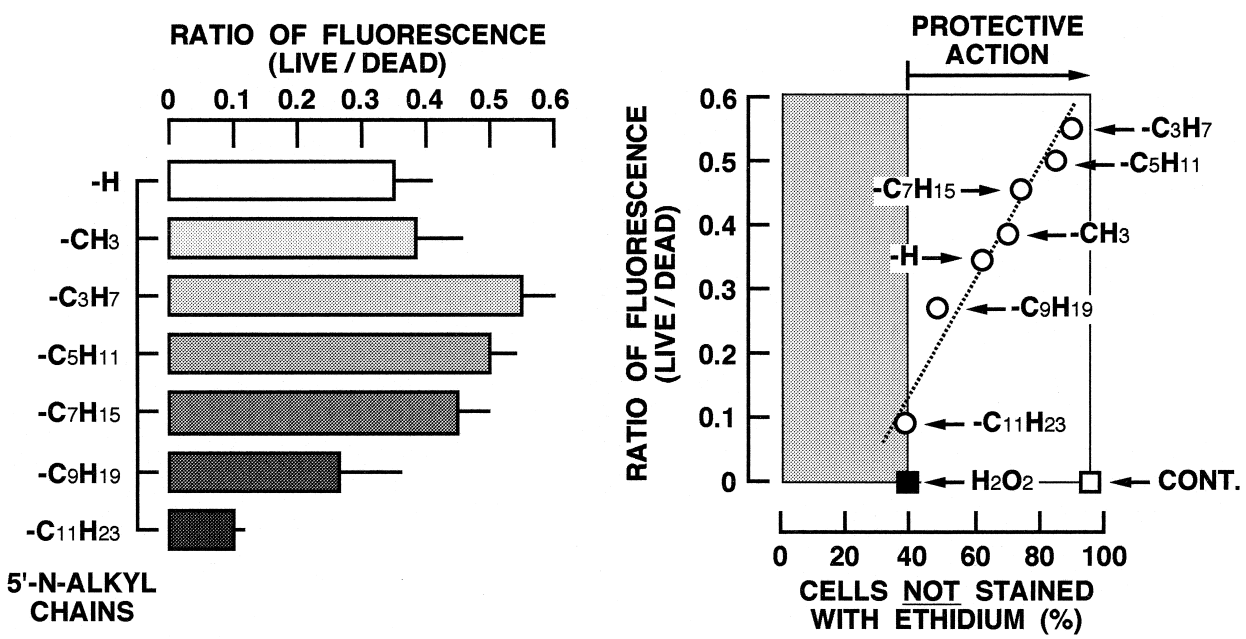

Fig. 7. Relation between protective action of 5′-*n*-alkyl curcumins and their cellular uptake. Left panel: the intensity ratios of fluorescence measured from live cells and dead cells treated with the 5′-*n*-alkyl curcumins (3 μM). Ratios are the fluorescence intensity of live cells/that of dead cells. Right panel: the relationship between the population of cells not stained with ethidium (similar results are shown in Fig. 3) and the fluorescence ratio of live cells/dead cells (shown in left panel).

using fluorescence, monitored at the wavelength of 530 ± 20 nm, of various 5′-alkylated curcumins at the concentration of 3 μM. As shown in Figs. 5 and 6, treatment of the cells with 5′-*n*-C_3H_7-curcumin increased the fluorescence in a time-dependent manner and fluorescence intensity reached steady state within 60 min. A further increase in fluorescence intensity of 5′-*n*-C_3H_7-curcumin was observed in the cells treated with 50 μM digitonin for disrupting the cell membranes. Similar phenomena were also observed in the case of 5′-*n*-C_9H_{19}-curcumin (Figs. 5 and 6). The times to reach the respective steady state levels increased as the length of 5′-*n*-alkyl chain of curcumin derivatives increased (Fig. 6). These results suggest that there are differences in the permeation of 5′-alkylated curcumins across the membranes, resulting in the differences in cellular concentrations of these compounds. Therefore, the fluorescence intensities of live cells treated with 5′-alkylated curcumins for 1 h were compared with those of the cells treated with 50 μM digitonin (the dead cells). Fig. 7 shows the ratios of fluorescence intensity of the curcumin derivatives obtained from live cells to that obtained from the dead cells. The ratio (fluorescence intensity of live cells/fluorescence intensity of dead cells) increased as the number of carbons in the 5′-*n*-alkyl chain of curcumin derivatives changed from 0 to 3. However, the ratio gradually decreased when the number of carbons changed from 3 to 6. Further increases in the number of carbons in the hydrocarbon chain (up to 11) greatly decreased the ratio.

4. Discussion

We have previously evaluated the protective actions of cassumunins A and B on thymocytes (Nagano et al., 1997) and brain neurons (Kanemaru et al., 1998) against oxidative stress. Both cassumunins at micromolar concentrations exerted the protective action on cells suffering from oxidative stress induced by H_2O_2, and the efficacies of cassumunins A and B on thymocytes were almost similar to their efficacies on brain neurons (Nagano et al., 1997; Kanemaru et al., 1998). Thus, the results obtained from thymocytes are useful to predict the results on other cells.

Oxidative stress of thymocytes was produced by 3 mM H_2O_2, a concentration that increased the number of cells stained with ethidium in a time-dependent manner, indicating a time-dependent decrease in cell viability. This decrease in cell viability was greatly attenuated by deferoxamine, an iron chelator (Fig. 4), suggesting the involvement of Fe^{2+} in the H_2O_2-induced decrease in cell viability. Since H_2O_2 and superoxide anion in the presence of Fe^{2+} produce hydroxyl radicals via the Feton reaction, the hydroxyl radical may be responsible for decreasing the viability of thymocytes treated with H_2O_2. Curcumin has been reported to possess antioxidant activities against reactive oxygen species such as the superoxide anion and the hydroxyl radical (Reddy and Lokesh, 1994; Ruby et al., 1995; Sugiyama et al., 1996). Since curcumin also exerts a protective activity on cells suffering from H_2O_2-induced oxidative stress (Nagano et al., 1997; Kanemaru et al., 1998), our experimental system using H_2O_2 is suitable for estimating the efficacy of 5′-alkylated curcumins.

The chemical structures of cassumunins A and B, 5′-$C_{12}O_2H_{15}$-curcumin and 5′-$C_{13}O_3H_{17}$-curcumin (Fig. 1, Masuda et al., 1998), include curcumin, which is known to have antioxidant activity (Sarma, 1976; Toda et al., 1985). The efficacy of cassumunins A and B to protect cells against the H_2O_2-induced oxidative stress was greater than that of curcumin (Nagano et al., 1997; Kanemaru et al., 1998). Therefore, the substitutes in the 5′ position of curcumin derivatives may be essential for the increased antioxidant activities of curcumin derivatives. The inhibitory actions of 5′-alkylated curcumins on lipid peroxidation in cell-free conditions increased as the length of 5′-*n*-alkyl chain increased. Such a potency order was not confirmed for the protective actions of 5′-*n*-alkyl curcumins on cells suffering from oxidative stress. Of the 5′-*n*-alkyl curcumins, 5′-*n*-C_3H_7-curcumin and 5′-*n*-C_5H_{11}-curcumin have a protective action much more potent than that of curcumin (Fig. 3). This may have been du, not only to the antioxidant activities increased by 5′-*n*-alkylation of curcumin, but also to increased permeation of these 5′-alkylated curcumins across the cell membranes and into the cells (Fig. 7). On the contrary, since the permeation of 5′-*n*-$C_{11}H_{23}$-curcumin into the cells was greatly reduced, this curcumin derivative did not exert any protective action on the cells suffering from the oxidative stress (Figs. 3 and 7). As shown in Fig. 7, there was a linear relationship between permeation of curcumin derivative and the efficacies of 5′-*n*-alkyl curcumins to protect the cells against oxidative stress. Thus, the protective actions of 5′-alkylated curcumins (Fig. 3) depended greatly on their permeations into the cells (Fig. 7). 5′-*n*-Alkyl group of curcumin derivatives plays an essential role in the modification of curcumin derivative permeation.

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