Antitumor and anti-invasive effects of diverse new macrocyclic lactones, alkylolides and alkenylolides, and their enhancement by hyperthermia

HIROSHI TANAKA[1], KATSUHIRO KAGEYAMA[1], KUMIKO KUSUMOTO[2], RYOKO ASADA[1] and NOBUHIKO MIWA[3]

[1]Osaka Butsuryo College, Sakai, Osaka 593-8328; [2]Department of Health Science, International Buddhist University, Habikino, Osaka 583-8501; [3]Laboratory of Cell-Death Control BioTechnology, Faculty of Life and Environmental Sciences, Prefectural University of Hiroshima, Shobara, Hiroshima 727-0023, Japan

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Abstract. Alkylolides and alkenylolides of 198-254 Da such as hexadecan-16-olide and 9-hexadecen-16-olide were chemically synthesized in the present study as new macrocyclic lactones that are structurally different from widespread natural macrocyclic lactones including bryostatin (887 Da) and rhizoxin (613 Da), and were investigated for antitumor activity to Ehrlich ascites tumor cells by mitochondrial dehydroganase-based WST-1 assay and dye-exclusion assay. Of the alkylolides having 12, 15 or 16 carbon-atoms (D12:0, P15:0 or H16:0) and alkenylolides having 15 or 16 carbon-atoms with a double bond (P15:1 or H16:1), H16:0 was the most carcinostatic when administered at 37˚C for 20 h, with cell deformation and microvillus disappearance as detected by scanning electron microscopy. The carcinostatic activity was increased markedly for H16:0 and P15:0 when the administration period was prolonged to 72 h, but was not enhanced by intramolecular introduction of a double bond for P15:1 or H16:1. Hyperthermia at 42˚C for 30 min additively intensified the carcinostatic activity for H16:0 and P15:0, but scarcely for D12:0, and intensified the alkenyloides P15:1 and H16:1 only upon the subsequent 72-h treatment. Invasion of human fibrosarcoma HT-1080 cells through the reconstituted basement membrane was inhibited by alkyl- and alkenylolides even after the short-term exposure at 25 μM for 3 h without diminishing the cell viability. H16:0 also exhibited the most inhibitory activity to tumor invasion in addition to the highest carcinostatic activity. Both inhibitions were promoted by combination with hyperthermia. Thus diverse alkyl-/alkenylolides, may be potent multi-applicable anticancer agents in terms of either dual inhibitory activities against both tumor progression and invasion or hyperthermia-combined therapy.

Correspondence to: Dr Katsuhiro Kageyama, Osaka Butsuryo College, 3-33, Otorikita-cho, Nishi-ku, Sakai, Osaka 593-8328, Japan
E-mail: kageyama@butsuryo.ac.jp

Key words: marocyclic lactone, alkylolide, alkenylolide, antitumor activity, hyperthermia, tumor invasion

Introduction

Natural macrocyclic lactones, such as bryostatin isolated from the marine bryozoan Bugula neritina (1) and rhizoxin from the plant pathogenic fungus *Rhizopus chinensis* (2) or *rhizopus sp* No.F-360 (3) have been reported to be potent anticancer agents in some preclinical murine models. Bryostatin exhibits antitumor activity against leukemia and melanoma (4,5), and rhizoxin against MH134 hepatoma (3). These macrocyclic lactones, i.e. bryostatin and rhizoxin are natural compounds showing a molecular weight of 887 and 613 Da (1,2).

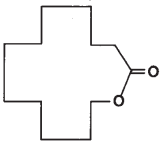
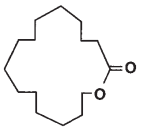
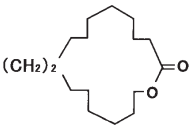
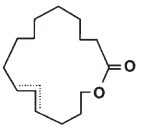
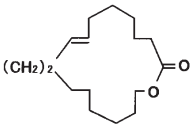
In the present study antitumor effects of alkylolides and alkenylolides, the lower molecular weight (198-254) of new macrocyclic lactones chemically synthesized, in contrast to the higher molecular weight (>600) of natural macrocyclic lactones, were examined from diverse viewpoints: i) enhanced carcinostasis in combination with hyperthermia, which inhibit tumor cell growth (6-8), and subsequent long-term culture, ii) morphological cellular degeneration, iii) anti-invasive activity as shown by diminished cell penetration through the reconstituted basement membrane.

Carcinostatic effect was evaluated with a decrease in mitochondrial dehydrogenase activity reflecting survival rate of cells as measured by WST-1 assay (9,10) and with a decrease in viable cells observed morphologically by trypan blue dye exclusion assay (11). Morphological cellular degeneration was observed by scanning electron microscopy (12). The ability of antitumor agents to inhibit tumor cell invasion into blood vessels prior to tumor metastasis is essential for clinic application (13).

Materials and methods

Materials. Alkylolides and alkenylolides were kindly provided by Soda Aromatic Co., Ltd. (Tokyo), were dissolved in ethanol, and stored frozen as test solutions. Details are shown in Table I.

Table I. Alkylolides and alkenylolides, macrocyclic lactones examined and their chemical structures.

Chemical name	Chemical formula	Abbreviation	Chemical structure
Dodecan-12-olide	$C_{12}H_{22}O_2$	D12:0	
Pentadecan-15-olide	$C_{15}H_{28}O_2$	P15:0	
Hexadecan-16-olide	$C_{16}H_{30}O_2$	H16:0	
10-Pentadecen-15-olide	$C_{15}H_{26}O_2$	P15:1	
9-Hexadecen-16-olide	$C_{16}H_{28}O_2$	H16:1	

Cells. Ehrlich ascites tumor cells (RCB: No.0142) were purchased from the Institute of Physical and Chemical Research (Tsukuba, Japan). Human fibrosarcoma cells HT-1080 were obtained from Japanese Cancer Research Resources Bank (JCRB, Tokyo). These cells were cultured in minimum essential medium (MEM) (Gibco Labs. Life Technologies, Inc., NY) supplemented with 10% fetal bovine serum (FBS) (Gibco Labs. Life Technologies, Inc.) culture medium.

Examination for carcinostatic effects: cell culture. The examination followed our developed system (10,14). Cells were suspended in the culture medium at a density of $2x10^5$ (culture for 20 h) or $2x10^4$ (culture for 72 h) cells/ml. An aliquot of the test solution was put in a test tube. After the solvent was evaporated by jet flow of nitrogen gas, culture medium was added to the residue (alkyl- or alkenylolide) and sonicated. The suspensions of cells and the test substance were mixed in a glass sample bottle (14 mm i.d. x 40 mm). The cells were finally diluted with adjustment to a cell density of $1x10^5$ or $1x10^4$ cells/ml. Then, the bottle was tightly covered with a plastic cap.

Hyperthermic treatment. The suspension was incubated at 37 or 42˚C for 30 min in a water bath (Model BT-23, Yamato Scientific Co. Ltd., Tokyo) and maintained by sequential culture in a humidified atmosphere of 5% CO_2 in air at 37˚C for 20 or 72 h.

Cell viability assay. Viability of cells was measured using two different methods: i) the redox indicator dye WST-1 (9) (Cell Counting Kit, Dojin Chemicals, Kumamoto, Japan) is more chromic according to mitochondrial dehydrogenase activity. The cultured cell suspension was transferred into a sampling tube and was centrifuged, the supernatant was completely removed from the tube, 110 μl of WST-1 (8%) per well was added to the cell precipitate, then suspended and transferred into each well of a 96-well microplate. After incubation at 37˚C for 1 h, the resultant formazan was determined by measuring the absorption at 450 nm with a plate reader (Benchmark, Bio-Rad Laboratories, CA). ii) Dye exclusion assay was performed as follows: the cultured cell suspension was treated as described above. Freshly prepared trypan blue dye solution in MEM (0.20%) of 60 μl was added to 60 μl of cell suspension, and microscopic counts of living (unstained) and dead (stained) cells were conducted on a hemocytometer (8,11).

Morphological observation of cells. Cells were treated in the presence of H16:0 alkylolide at 37 or 42˚C for 30 min and cultured for 20 h in a CO_2 incubator. Specimens were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.2) at room temperature for 2 h. The specimens were then placed in 0.1 M phosphate buffer overnight, and postfixed with 1% osmium tetroxide for 2 h, then washed in re-distilled water (RWD) followed by

dehydration through a graded series of ethanol. For scanning electronic microscopic (SEM) study, samples were transferred to tert-butyl alcohol, and dried using a freeze drier (ES-2030, Hitachi, Tokyo), sputter-coated with gold-palladium, and examined in a Hitachi S-2460N scanning electronic microscope operated at 5 kV (12).

Tumor cell invasion assay. To each well of a 24-well cuture plate, 500 μl of 10% FBS-MEM was added, and then a Chemotaxicell (Invasion Chamber, Kurabo Industries, Ltd., Osaka) having a porous resin membrane (pores with an 8-μm diameter) precoated with Matrigel (Wako Pure Chemical Industries, Ltd., Osaka) was placed. In addition, 200 μl ($2x10^5$ cells) of the above-mentioned suspension of human fibrosarcoma HT-1080 cells treated at 37 or 42˚C for 1 h in the presence of alkyl- and alkenylolides (25 μM) was added and the plate was incubated for 3 h. After incubation, the Chemotaxicell was removed, and after eliminating the supernatant and MatrixGel inside the Chemotaxicell, cells on the lower side of resin membrane of the Chemotaxicell were fixed and stained using DifQuik (Green Cross, Osaka). After the resin membrane was removed from the Chemotaxicell and fixed with the back surface on top in a slide glass, and the number of stained cells was counted under a microscope (13).

Statistics. Statistical differences were analyzed by Student's t-test.

Results

Carcinostatic effects of alkylolides and alkenylolides on short-term (20 h) or long-term (72 h) cultures. Diverse species of alkyl-/alkenylolides were added to EAT cells, the samples were heated in a water bath at 37˚C for 30 min and were maintained by sequential culture in a humidified atmosphere of 5% CO_2 in air at 37˚C for 20 h. The carcinostatic effects were measured using the WST-1 assay (Fig. 1A). Taking the cell viability of the control in the absence of alkyl-/alkenylolide as 100%, the viability of the experimental samples at a dose of 100 μM was evaluated. Hexadecane-16-olide (H16:0), pentadecane-15-olide (P15:0), and hexadecene-16-olide (H16:1) were carcinostatic, giving a cell survival rate of 3.9±2.2% (P<0.0001), 31.7±6.7 (P<0.0001), and 76.2±7.5 (P<0.0115) at 37˚C, respectively. The other samples, dodecane-12-olide (D12:0) and pentadecene-15-olide (P15:1) were almost ineffective.

To examine the effect of long-term exposure of alkyl-/alkenylolides, the cells were further cultured at 37˚C for 72 h and the carcinostatic activity was measured by WST-1 assay (Fig. 1A). At a dose of 50 μM, H16:0 and P15:0 diminished the cell viability to 1.9±0.6% (P<0.0001) and 23.6±4.3% (P<0.0001), respectively, corresponding to the values at 100 μM in the culture for 20 h, but the others exhibited slight activity or none. Treatment with H16:0, even at a dose as low as 25 μM, decreased the cell viability to 5.6±3.8% (P<0.0001), whereas P15:0 was not effective at the same dose (almost inactive at 10 μM).

Carcinostatic activities of alkyl-/alkenylolides to the cells treated in the same manner as described above was also measured by trypan blue dye exclusion assay (Fig. 1B). It was shown that H16:0, P15:0, and H16:1 at a dose of 100 μM diminished the cell viability to 14.0±8.9% (P<0.0001), 42.2±8.5% (P<0.0001), and 58.2±1.4% (P<0.0001) versus that of the control, whereas P12:0 and H15:1 were not markedly carcinostatic.

Viability of the cell cultured for 72 h after the hyperthermic treatments was diminished by H16:0 and P15:0 to 0.00% and 46.5±6.5% (P<0.0001) at 50 μM, respectively, whereas the other alkyl-/alkenylolides were slightly or scarecely carcinostatic. Treatment with H16:0, even at a dose of 25 μM, decreased the cell viability to 1.0±1.2% (P<0.0001). The results roughly coincided with those obtained by WST-1 assay.

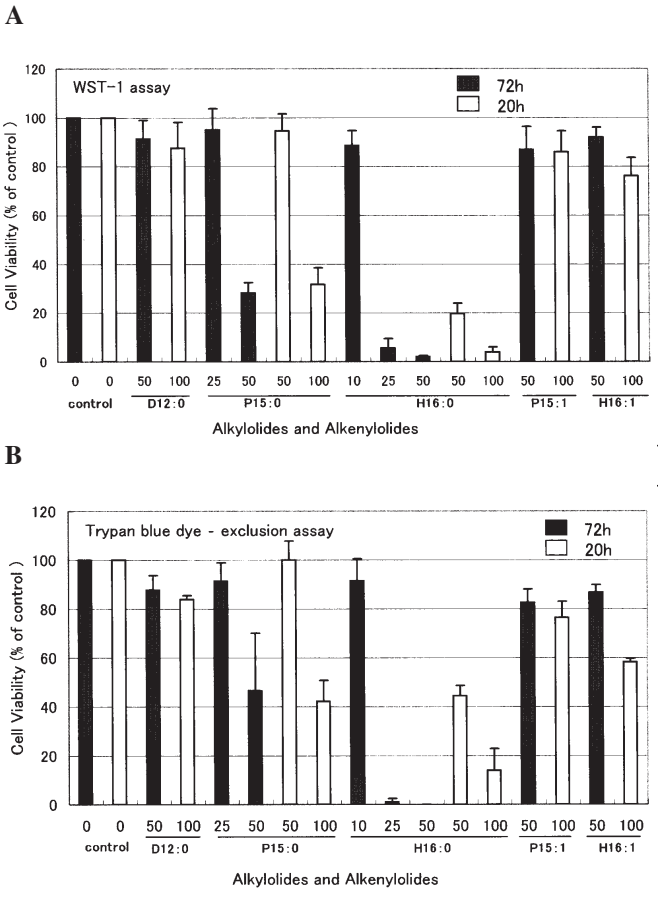


Figure 1. (A) Short- and long-term carcinostatic effects of alkylolides and alkenylolides on Ehrlich ascites tumor (EAT) cells as measured by mitochondrial dehydrogenase-based WST-1 assay. Cells were seeded at a density of $1x10^5$ (20 h cultures) or $1x10^4$ (72 h cultures) cells/ml, incubated in the presence of each alkyl-/alkenylolide at a dose of 10-100 μM at 37˚C for 30 min and further maintained by sequential culture at 37˚C for 20 or 72 h. Viability of the cells was measured by the absorption at 450 nm in WST-1 assay. The absorbance of cells treated in the absence of alkyl-/alkenylolide at 37˚C was 1.432±0.437 or 2.063±0.321 (the control value), respectively. Data shown represent the means ± SEM for quadruplicate measurements as percentages of the control value. (B) Short- and long-term carcinostatic effects of alkylolides and alkenylolides on the EAT cells as measured by trypan blue dye exclusion assay. Cells were treated as described in (A). Viability of the cells was evaluated by the trypan blue dye exclusion assay. The number of viable cells in the absence of alkyl-/alkenylolide at 37˚C was $2.08±0.71x10^5$ or $1.90x10^5$ (the control value). Experimental values represent the means ± SEM for triplicate measurements as percentages of the control value.

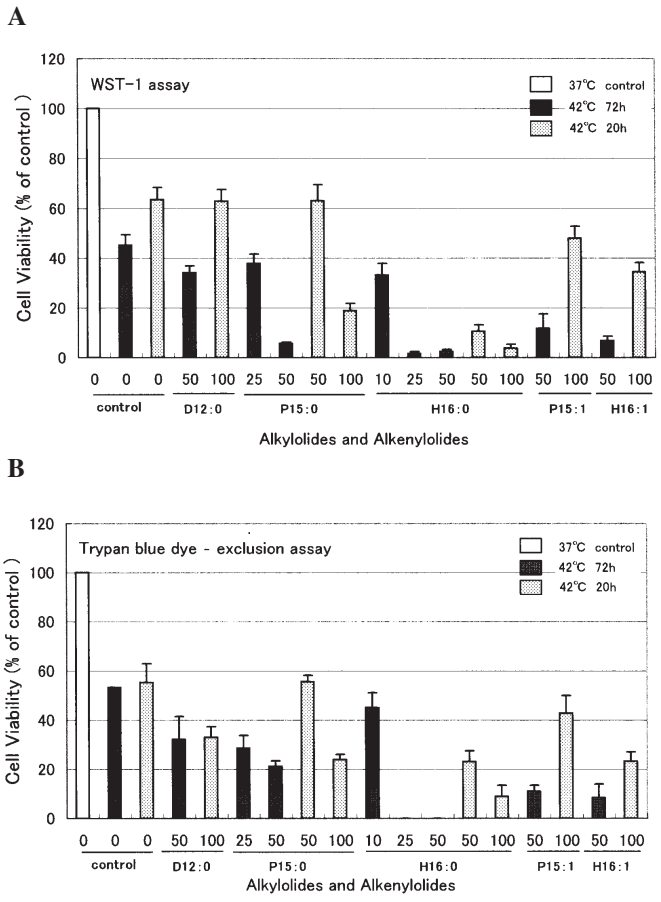


Figure 2. (A) Short- and long-term carcinostatic effects of alkylolides and alkenylolides on Ehrlich ascites tumor (EAT) cells in combination with hyperthermia (as measured by mitochondrial dehydrogenase-based WST-1 assay). Cells were seeded at a density of $1x10^5$ (20 h cultures) or $1x10^4$ (72 h cultures) cells/ml, incubated in the presence of each alkyl-/alkenylolide at a dose of 10-100 μM at 42˚C for 30 min and further maintained by sequential culture at 37˚C for 20 or 72 h. Viability of the cells was measured by the absorption at 450 nm in WST-1 assay. The absorbance of cells treated in the absence of alkyl-/alkenylolide at 37˚C was 1.432±0.437 or 2.063±0.321 (the control value), respectively. Data shown represent the means ± SEM for quadruplicate measurements as percentages of the control value. (B) Short- and long-term carcinostatic effects of alkylolides and alkenylolides on the EAT cells in combination with hyperthermia (as measured by trypan blue dye exclusion assay). Cells were treated as described in (A). Viability of the cells was evaluated by the trypan blue dye exclusion assay. The number of viable cells in the absence of alkyl-/alkenylolide at 37˚C was $2.08±0.71x10^5$ or $1.90x10^5$ (the control value). Experimental values represent the means ± SEM for triplicate measurements as percentages of the control value.

Effect of hyperthermia on carcinostatic effects of alkyl-/alkenylolides. Viability of EAT cells cultured for 20 h after the treament at 42˚C for 30 min was measured by WST-1 assay (Fig. 2A). Hyperthermia alone at 42˚C diminished the viability to 63.5±4.9% (P<0.0001) versus that of the control at 37˚C. The viabilities at a dose of 100 μM were in the following inverse order: H16:0 [6.0±2.4% (P<0.0001)] < P15:0 [29.6±4.7% (P<0.0001)] < H16:1 [54.2±8.4% (P<0.0001)] < P15:1 [75.4±6.6% (P<0.0076)] indicating the carcinostasis exceeding over that due to hyperthermia alone (100±7.3%). At a dose of 50 μM, only H16:0 diminished the cell viability to 16.7±3.9% (P<0.0001) whereas P15:0 was quite ineffective.

In long-term culture period for 72 h after the hyperthermic treatment, H16:0 diminished the viability at a dose of 25 μM almost completely [3.8±1.8% (P<0.0001)], and was effective even at 10 μM [73.4±10.6% (P<0.0296)]. P15:0 decreased to 12.6±0.9% (P<0.0001) at 50 μM, but was not effective at 25 μM, indicating a carcinostatic activity weaker than that of H16:0 also upon combination with hyperthermia. The alkenylolides H16:1 and P15:1 at 50 μM, non-effective at 37˚C, markedly reduced the viability to 15.1±4.0% (P<0.0001) and 25.9±13.1% (P<0.0001) versus that of the control, respectively. In contrast, D12:0 was the only examined compound that was not affected by hyperthermia at the same dose.

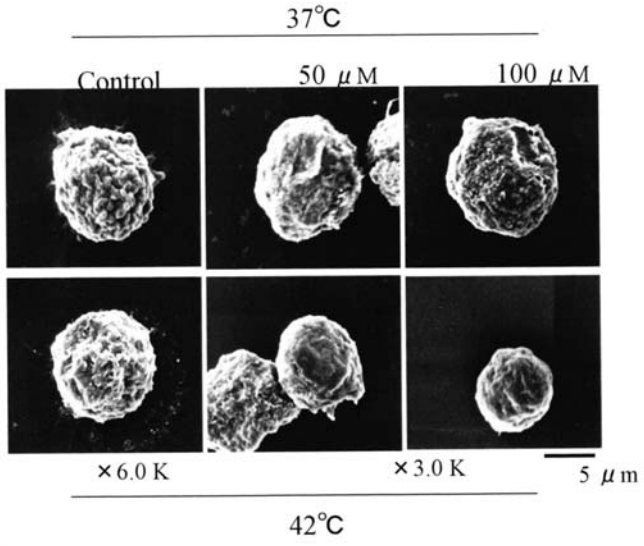


Figure 3. Scanning electron micrographs of EAT cells that were exposed to hexadecane-16-olide (H16:0). Cells were incubated in the presence of H16:0 at a dose of 50 or 100 μM at 37 or 42˚C for 30 min and maintained by sequential culture at 37˚C for 20 h, fixed and washed as conventionally conducted. Cells were fixed again with 1% osmic acid, washed and dehydrated. Cells were coated with ions after lyophilization, and cell shape was observed under a scanning electron microscope (x6.0 K, x3.0 K).

Carcinostatic effects of alkyl-/alkenylolides (at 100 μM) on the cells cultured for 20 h after hyperthermia at 42˚C, was measured by trypan blue dye exclusion assay (Fig. 2B). H16:0, P15:0, and H16:1 enhanced diminution of cell viability to 16.1±8.2% (P<0.0001), 43.5±3.6% (P<0.0001), and 42.0±6.7% (P<0.0001) versus that (100%) of hyperthermia alone, respectively. The others were scarcely carcinostatic at the same dose at 42˚C.

At 72-h culture after the treatment H16:0, P15:0, H16:1 and H15:1 further diminished cell viability to 0.0% (P<0.0001), 25.2±2.7% (P<0.0001), 15.8±10.5% (P<0.0001) and 20.6±4.5% (P<0.0001) versus that (100%) of hyperthermia alone, respectively. These results were approximately consistent with those by WST-1 assay.

Morphological changes in tumor cells observed by scanning electron microscopy. The carcinostatic effects were shown to be the greatest for H16:0 out of diverse samples, and morphological changes in EAT cells were therefore investigated using H16:0. Scanning electronic microscopy elucidated the morphological aspects (Fig. 3) of the cells treated with H16:0 at 37 or 42˚C for 30 min and subsequently cultured for 20 h. Hyperthermia at 42˚C induced a drastic damage to the cell membrane surface including the disappearance of microvilli

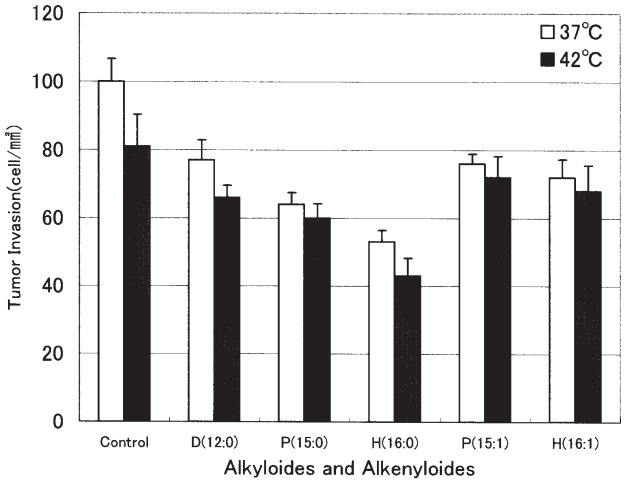


Figure 4. Inhibitory activity of alkylolides and alkenylolides against invasive activity of human fibrosarcoma HT-1080 cells. To a Chemotaxicell in a 24-well plate, 200 μl of 5 or 20 μM of alkyl-/alkenylolides and HT-1080 cell suspension ($2x10^5$ cells) were placed, and incubated for 1 h. The cells that invaded through the reconstituted basement membrane were fixed and stained. The stained cells were counted under a microscope. Data shown are typical of three independent experiments that were conducted in triplicate; SD is represented by the bar.

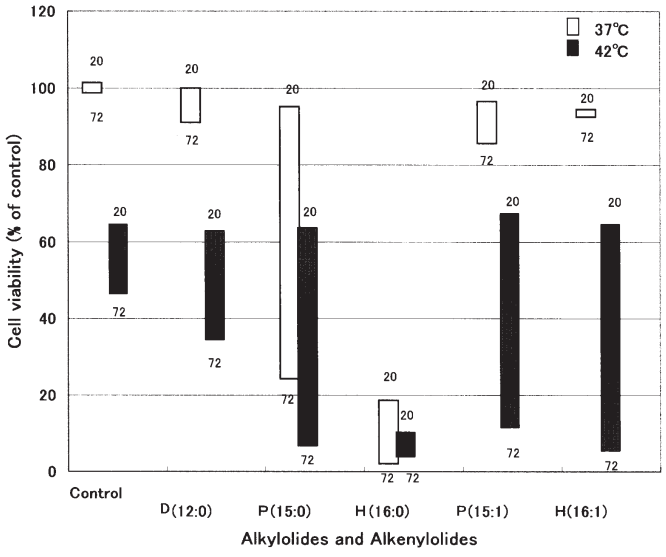


Figure 5. Effects of hyperthermia and sequential-culture period on the carcinostatic activities of diverse alkylolides and alkenylolides. The effects were calculated based on the values obtained at a dose of 50 μM (see Fig. 1A and Fig. 3A). An open bar represents the value at 37˚C and a closed bar that at 42˚C. The top and bottom termini of a bar exhibit the cell viability in sequential culture for periods of 20 and 72 h, respectively. Longer bars show that the long-term culture promoted the cacinostatic activity more markedly than the short-term culture did. An open bar exhibits the value at 37˚C and a closed bar exhibits that at 42˚C.

in contrast to the intact aspects together with normal microvilli for untreated control. Furthermore, treatment with H16:0 induced cell destruction at 37˚C, and significantly enhanced markedly together with even cell fragmentation at 42˚C. Frequency of the above-mentioned abnormal cell morphology was more marked for the H16:0- and/or hyperthermia-treated cells than for the untreated control cells.

Effects on tumor cell invasion. The effect of diverse alkyl-/alkenylolides on tumor cell (human fibrosarcoma HT-1080 cells) invasion is shown in Fig. 4. In non-additive (control) sample, 541 of total cells ($2x10^5$) passed through the porous resin membrane of the Chemotaxicell. Even the exposure of short-period (3 h) at low concentrations of 25 μM, invasion was suppressed by all alkyl-/alkenylolides in order of H16:0>P15:0>D12:0>H16:1>P15:1 in intensity. The suppressive activity was augmented in relation to the increase of their carbon atom, resulting the increase of their carcinostatic activity. H16:0 exhibited the highest suppressive activity, which reduced to 53.0±3.4% ($P<0.0001$) of control value (100%). The value of D12:0 exhibiting the lowest activity was 77.0±5.8% ($P>0.0044$). At 42˚C, H16:0 and P15:0 reduced to 52.9±6.5% ($P<0.0002$) and 73.5±5.2% ($P<0.0179$) versus that (100%) of hyperthermia alone, respectively, but not other alkyl-/alkenylolides.

Discussion

Antitumor activity has been found in several macrocyclic lactones such as bryostatin and rhizoxin contained in various fungi (1-3). These natural compounds show a molecular weight of several hundred Da. In the present study, carcinostatic activity was detected in diverse chemically synthesized alkyl-/alkenylolides with molecular weights of 198-254 lower than those of the above-mentioned macrocyclic lactones. They inhibit the mitochondrial dehydrogenase activity and the growth of Ehrlich ascites tumor cells strongly in the order of H16:0>P15:0>H16:1>P15:1>D12:0 (Figs.1 and 2). This order in degrees of carcinostatic activity was approximately consistent regardless of differences in bioassay methods, treatment periods (20 or 72 h) and treatment temperatures (37 and 42˚C). Unexpectedly, it was found that: i) the carcinostatic activity of alkylolides diminishes along with decreasing number of carbon atoms. Chain-length shortening of H16:0 to P15:0 by only one methylene unit causes drastic attenuation of the carcinostatic ability. ii) saturated alkylolides such as H16:0 and P15:0 exceed over the corresponding unsaturated alkenylolides such as H16:1 and P15:1. These theories are in contrast to those applicable to fatty acids (14) and fatty alcohol (15).

Effects of hyperthermia and sequential culture period on the carcinostatic activities of diverse alkyl-/alkenylolides were illustrated based on the cell viabilities obtained at a dose of 50 μM (Fig. 1A and Fig. 2A) (Fig. 5). The diverse alkyl-/alkenylolides can be classified as follows: i) H16:0 possesses the strongest carcinostatic activity regardless of hyperthermia and sequential long-term culture. ii) H15:0 enhances its activity more markedly by sequential long-term culture rather than by hyperthermia. iii) H12:0 scarcely enhances by either the treatment, indicating the smallest carcinostatic activity. iv) H16:1 and H15:1 enhance their activity only by dual consecutive treatments of sequential long-term culture after hyperthermia, but do not enhance by a single treatment of long-term culture or hyperthermia.

Inhibition of invasion of tumor cells using a blood vessel model *in vitro* is considered to reflect prevention of permeation to vein in the first step of metastasis (13). Alkyl-/alkenylolides could inhibit invasive action of human fibrosarcoma HT1080 cells even after the short-term exposure at

25 μM for 3 h not diminishing cell viability. The inhibitory activity is elevated according to the increase of their carbon atoms. Accordingly, H16:0 exhibited the highest inhibitory activity as well as the highest carcinostatic activity. These effects are promoted by hyperthermia.

When mechanisms of the antitumor effects are considered, natural macrocyclic lactones, such as bryostatin and rhizoxin may be due to stimulating the transcription of cyclooxygenase-2 (16), activating protein kinase C (17), and inhibiting cell proliferation (16), the mitosis in cells and tubulin polymerization (18).

The authors have regarded intracellular permeation of alkyl-/alkenylolides as a cause of cytotoxic actions. Fatty acids (19), hydroxy fatty acids (10), and fatty alcohols (15) that are incorporated into the cells (detected by gas-liquid chromatography) inhibit DNA synthesis (19,15), mitochondrial dehydrogenase activity and growth of tumor cells (10). δ-alkyllactone is also incorporated into tumor cells (23). However, the alkyl-/alkenylolides used in the present study could not be detected in the cells, whereas they were detected in the extracellular liquid. However, they could not be detected in the cells. Therefore, their carcinostatic effects may be due to their surface activity towards the cell membrane. Hyperthermia is known to inhibit the DNA synthesis in tumor cells (20-22) and to diminish the tumor cell viability by combination with anti-cancer agents. As morphologically observed by SEM, the injury of cell membrane surface and the deformation of cells can be regarded as typical symptoms indicative of cytotoxic action of H16:0 and hyperthermia.

Of the new macrocyclic lactones, hexadecane-16-olide (H16:0) exhibits the most carcinostatic activity and tumoricidal action; the activity of hexadecen-16-olide (H16:1) is considerably enhanced by hyperthermia and can destruct the tumor-tissue specifically by means of hyperthermia locally applied to the target tumor.

Thus, some alkyl-/alkenylolides are more attractive as potent antitumor regimens in terms of reduction of both the side-effect towards the normal tissues and the lethal metastasis occurring secondarily after application of some conventional cytotoxic anticancer agents.

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