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PROMOTIVE EFFECTS OF HYPERTHERMIA ON THE CYTOSTATIC ACTIVITY TO EHRLICH ASCITES TUMOR CELLS BY DIVERSE DELTA-ALKYLLACTONES

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Aim: **To evaluate promotive effects of hyperthermia on antitumor activity of new delta-alkyllactones (DALs) of low molecular weight (184–254 Da), chemically synthesized, which are different from natural macrocyclic lactones of high molecular weight (348–439 Da), such as camptothecin and sultriecin.** ***Methods:*** **A suspension of Ehrlich ascites tumor (EAT) cells was mixed with a DAL in a glass tube, heated at 37 or 42 °C for 30 min in a water bath, and cultured at 37 °C for 20 or 72 h. Cell viability was measured by the mitochondrial dehydrogenase- based WST-1 assay. DALs incorporated into EAT cells was extracted and measured by gas-liquid chromatography.** ***Results:*** **The reduction of cell viability by DALs was markedly enhanced upon the treatment at 42 °C compared to that at 37 °C. At 37 °C, delta-hexadecalactone (DH16 : 0) and delta-tetradecalactone (DTe14 : 0) displayed cytostatic activity (at 100 μM survival level: 20.7%, 66.1%; at 50 μM — 41.2%, 82.4%, respectively). Their activity was more marked at 42 °C (at 100 μM 10.6%, 27.6%; at 50 μM 30.6, 37.5 %, ibid). The other DALs, delta-undecalactone (DU11 : 0), delta-dodecalactone (DD12 : 0), and delta-tridecanolactone (DTr13 : 0) were almost ineffective. Evaluation of survival rate in the cells treated for 30 min by DALs with the next culturing of EAT cells for 72 h resulted in the enhanced carcinostatic activity of DH16:0 and DTe14:0 even at concentrations as low as 25 μM at either 37 °C (18.5%, 78.5%, ibid) or 42 °C (5.0%, 42.0%, ibid), but the others exhibited slight activity or none. DH16 : 0 was effective at either 37 °C (36.0%) or 42 °C (23.0%) even at a lower dose of 10 μM. At the same time only the most cytostatic DH16 : 0 was incorporated into EAT cells and the rate of incorporation was more at 42 °C than at 37 °C.** ***Conclusion:*** **Delta-hexadecanolactone (DH16 : 0) exhibited the most cytostatic effect that was significantly enhanced by hyperthermia. It allows to consider it as a potent antitumor agent, especially in combination with hyperthermia.**
Key Words: **delta-alkyllactone, delta-hexadecalactone, hyperthermia, antitumor activity.**

For many years, delta-lactones (DLs), produced from plant or fungi, such as camptothecin [1, 2], kazusamycin [3], sultriecin [4], have been studied on their antitumor activity.

In contrast to these natural DLs (348–439 Da) of high molecular weight, we found anti-tumor effects of the delta-alkyllactones (DALs) (184–254) of low molecular weights, which are structurelly simple and chemically synthesized [5].

Carcinostatic effect was measured by the mitochondrial dehydrogenase-based WST-1 assay [6–8]. Among all the DALs, the most carcinostatic delta-hexadecalactone (DH16 : 0) at the dose of 100 μM diminished the viability of Ehrlich ascites tumor (EAT) cells cultured at 37 °C for 20 h to 20.7% of the control (100%) in the absence of DAL. When the cells further cultured for 72 h, DH16 : 0 decreased cell survival rate to 0.8% at 50 μM and to 18.5% even at low dose of 25 μM [5].

Hyperthermia is receiving attention as effective mean in combined cancer treatment. Exposure of cultured tumor cells to temperatures above 41 °C results in inhibition of the DNA synthesis [9, 10] and proliferation of tumor cells [11, 12].

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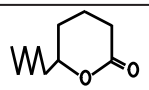
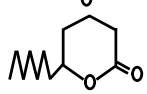
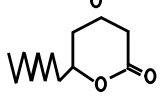
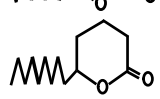
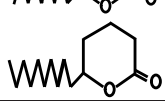
Abbreviations used: DAL – delta-alkyllactone; EAT – Ehrlich ascites tumor; FBS – fetal bovine serum; MEM – minimum essential medium; PUFA – polyunsaturated fatty acid; ROS – reactive oxygen species.

In the present study, in order to exceed beyond the carcinostatic effect of DALs alone, hyperthermia was combined. We studied whether their carcinostatic effect on EAT cells is markedly enhanced by a combination with hyperthermia. Furthermore, the relationship between the carconostatic activity of the DALs and the intracellular uptake determined by gas-liquid chromatography (GLC) [13, 14] and whether hyperthermia increase amount of the intracellular accumulation was investigated.

MATERIALS AND METHODS

Materials. DALs kindly provided by Soda Aromatic Co., Ltd. (Tokyo, Japan), were dissolved in ethanol, and stored in a freezer as test solutions (Table).

Table. Delta-alkyl lactones examined and their chemical structures

Chemical name	Chemical formula	Abbreviation	Chemical structure
Delta-undecalactone (5-Undecanolide)	$C_{11}H_{20}O_2$	DU11 : 0	
Delta-dodecalactone (5-Dodecanolide)	$C_{12}H_{22}O_2$	DD12 : 0	
Delta-tridecalactone (5-Tridecanolide)	$C_{13}H_{24}O_2$	DTr13 : 0	
Delta-tetradecalactone (5-Tetradecanolide)	$C_{14}H_{26}O_2$	DTe14 : 0	
Delta-hexadecalactone (5-Hexadecanolide)	$C_{16}H_{30}O_2$	DH16 : 0	

Cells. EAT cells (RCB: No. 0142) were purchased from the Institute of Physical and Chemical Research (Tsukuba, Japan). The cells were cultured in minimum essential medium (MEM) (GIBCO Life Technologies, Inc., NY, USA) supplemented with 10% fetal bovine serum (FBS) (GIBCO Life Technologies).

Cell culture and exposure of hyperthermic treatment. Cells were suspended in the culture medium at a density of 2×10^5 (20 h cultures) or 2×10^4 (72 h cultures) cells/ml. Aliquots microlitter of the test solution were added to test tubes. After the solvent was evaporated by a jet flow of nitrogen gas, culture medium was added to a residue (DAL), and sonicated. The suspensions of cells and the test substance were mixed in a glass sample bottle (14 mm i. d. × 40 mm). The cells were finally adjusted to a cell density of 1×10^5 or 1×10^4 cells/ml.

Cells in a tightly stopped tube were heated, at 37 °C or 42 °C controlled within ± 0.05 °C for 30 min in a water bath (Model C-650, Taiyo Scientific Industrial Co., Ltd., Japan) [10]. Then, the bottles were covered with a glass cap. The suspension was cultured in a humidified atmosphere of 5% CO_2 in air at 37 °C for 20 h, almost equal to the cell cycle period, or for 72 h for a longer exposure to DAL.

Cell viability assay. Cell viability was measured by the redox indicator dye WST-1, which is related to the degree of mitochondrial dehydrogenase activity [6, 7] (Cell counting kit Dojin Chemicals, Kumamoto, Japan). The cultured cell suspension was transferred to a sampling tube and centrifuged. The supernatant was completely removed from the tube, 110 µl of WST-1 (8%) per well was added to each cell precipitate, which resuspended, and transferred into each well of a 96-well microplate. The resultant diformazan formation was determined by measuring the absorption at 450 nm with a plate reader (Benchmark, Bio-Rad Laboratories, CA, USA) after incubation at 37 °C for 1 h.

DAL accumulation in cells. Cells were suspended in culture medium, and adjusted to a final cell density of 2×10^5 cells/ml. The DALs were dispersed at a final concentration of 25 µM by sonication. The cell suspension and DAL emulsion (7 ml each) were mixed in a glass sample vial (27 mm i. d. x 65 mm). The cells were finally adjusted to a cell density of 2×10^5 cells/ml. Cells in a tightly stopped tube were heated, at 37 °C or 42 °C controlled within ± 0.05 °C for 30 min in a water bath [13]. Then, the bottles were covered with a glass cap. The heated suspension was incubated in a humidified atmosphere of 5% CO_2 in air at 37 °C for 1 h. The suspension was then centrifuged, and the supernatant was withdrawn with a pipette. The Pellet (packed cells) was rinsed twice with 2 ml of fresh culture medium, and homogenized for 1 min after removing the supernatant as much as possible with a microliter syringe and adding 1 ml chloroform. The mixture was poured into a glass tube (5.5 mm i. d. x 200 mm long) sealed with cotton, and the solvent was evaporated by a nitrogen gas stream. Chloroform was added to the residue (DAL) and the sample (3 µl) was analyzed with a gas chromatograph equipped with a flame ionization detector (GC-6AM; Shimadzu Seisakusho, Kyoto, Japan) [13, 14].

Statistics. The statistical differences were examined by the Student's *t*-test.

RESULTS

Carcinostatic effects of DALs and/or hyperthermia on short term (20 h)-culture. Several *DALs* were added to EAT cells, which were cultured for 20 h. The carcinostatic effects were measured using the WST-1 assay (Fig. 1). Taking the viability of the control in the absence of DAL as 100%, viability of the experimental samples at dose of 50 µM or 100 µM were evaluated. Among all the DALs studied, delta-hexadecalactone (DH16 : 0) and delta-tetradecalactone (DTe14 : 0) were effective, giving a cell survival rate of 20.7 ± 2.2 % ($P < 0.0001$) and 66.1 ± 4.5% ($P < 0.0001$), at 100 µM, respectively. The other DALs, delta-undecalactone (DU11 : 0), delta-dodecalactone (DD12 : 0), and delta-tridecanolactone (DTr13 : 0) were almost ineffective. DH16 : 0 and DTe14 : 0, even at 50 µM, decreased cell viability to 41.2 ± 2.5% ($P < 0.0001$) and 82.4 ± 5.4 ($P < 0.0263$), respectively.

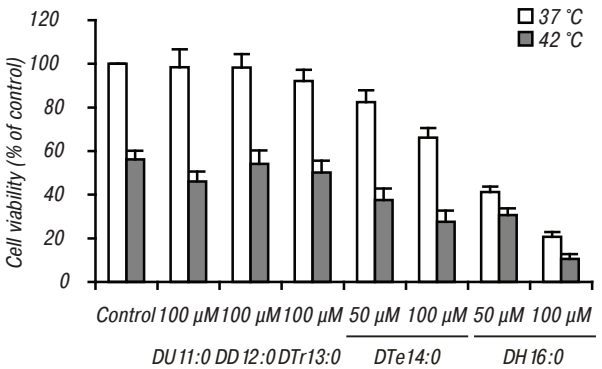


Fig. 1. The carcinostatic effects of delta-alkyllactones (DALs) on Ehrlich ascites tumor (EAT) cells cultured for 20 h after the treatment at 37 °C or 42 °C. Cells were seeded at a density of 1 x 10^5 cells/mL, and cultured in the presence of each DAL at a dose of 50 or 100µM at 37 °C for 20 h. Viability of the cells was measured by the absorption at 450 nm with a DAL in WST-1 assay. The absorbance of cells treated for 20 h in the absence of DAL was 1.432 ± 0.437 (control values). Data shown represent the means ± SEM for quadruplicate measurements as the percentages of the control value

The heat treatment alone at 42 °C diminished cell viability to 56.2 ± 3.9% ($P < 0.0001$) compared to control EAT cells at 37 °C (100%). At 42 °C the diminution of cell viability was also enhanced by the administrations of H16 : 0 and Dte14 : 0 at 100 µM to 18.9 ± 3.9% ($P < 0.0001$), 49.1 ± 9.1% ($P < 0.0001$), and at 50 µM to 54.4 ± 5.7% ($P < 0.0001$) and 66.7 ± 9.6% ($P < 0.0024$), respectively, but the other DALs exhibited slight or no activity.

To examine the effect of long-term exposure of diverse DALs, the cells were further cultured at 37 °C for 72 h and the carcinostatic activity was measured by WST-1 assay (Fig. 2). At a dose of 50 µM, DH16 : 0 and DTe14 : 0 diminished the cell viability to 0.8 ± 0.1% ($P < 0.0001$) and 68.0 ± 8.2% ($P < 0.0067$), respectively, more effective than the values at 100 µM in the culture for 20 h,

but the others exhibited slight activity or none. DH16 : 0 and DTe14 : 0, even at a dose of 25 μM, decreased cell survival rate to 18.5 ± 3.7% ($P < 0.0001$) and 78.5 ± 7.2% ($P < 0.0405$) and DH16 : 0, even at a extremely low dose of 10 μM, to 36.0 ± 6.3% ($P < 0.0001$), whereas DTe14 : 0 was not effective at same dose. Hyperthermia alone at 42 °C decreased cell viability to 57.9 ± 6.0% ($P < 0.0001$) of the control value (at 37 °C). Carcinostatic effects of DH16 : 0 and DTe14 : 0 further enhanced by hyperthermia and long term-culture. Their effects at 25 μM were enhanced to 7.4 ± 3.6% ($P < 0.0001$) and 72.7 ± 9.2% ($P < 0.0001$) of the value (100%) in hyperthermia alone, respectively. Furthermore, DH16 : 0 even at 10 μM, to 39.7 ± 7.8% ($P < 0.0001$), whereas DTe14 : 0 was not effective at same dose. The others exhibited slight activity or none even at high dose of 50 μM.

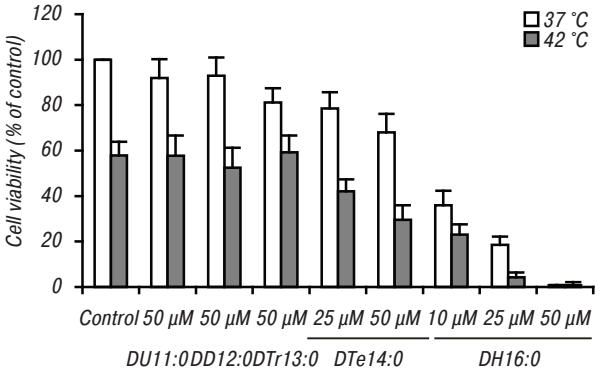


Fig. 2. The carcinostatic effects of delta-alkyllactones (DALs) on Ehrlich ascites tumor (EAT) cells cultured for 72 h after the treatment at 37 °C or 42 °C. Cells were seeded at a density of 1 x 10^4 cells/ml, and cultured in the presence of each DAL at a dose of 10, 25 or 50 μM at 37 °C for 72 h. Viability of the cells was measured by the absorption at 450 nm with a DAL in WST-1 assay. The absorbance of cells treated for 72 h in the absence of DAL was 2.063 ± 0.321 (control values). Data shown represent the means ± SEM for quadruplicate measurements as the percentages of the control value

Intracellular accumulation of DALs added to tumor cells. The more carcinostatic DALs tend to be taken up and accumulated more abundantly in the tumor cells [15], and accordingly the accumulation was measured in the cells subjected to therapy of DALs. Cell extracts were quantified by GLC (see Fig. 2). Intracellular uptake was markedly detected for DH16 : 0 (4.2 μg/total cells or 1.5 pg/cell), which was outstandingly more carcinostatic than the other DALs. The value corresponded to 4.7% of the total amount of DH16 : 0 added to the cell culture. At 42 °C, the uptake of DH16 : 0 was enhanced to 5.0 μg/total cells (1.8 pg/cell) and corresponded to 5.6% of the total amount of administration. The other DALs, however, were scarcely detected in cells at 37 °C or 42 °C.

DISCUSSION

The higher molecular weights of natural delta-alkyllactone, such as, Camptothecin [1, 2] and Kazusamycin [3] have been reported to show antitumor activity. In the present study, the carcinostatic effects of the low molecular weight of simple delta-alkyllactones (DALs) synthesized chemically were assessed by assays for mitochondrial dehydrogenase activity. The results revealed that DH16 : 0 had the most potent carcinostatic action. With regard to carcinostatic action, the present results suggest that the activity elevates with increasing carbon atom in contrast to the conventional concept applicable to fatty acids [10] and fatty alcohols [16].

Their carcinostatic effects were markedly enhanced with elongating the cell culture period. DH16 : 0 at 50 μM diminishes perfectly the cell viability by the exposure for 72 h, whereas to 41.2% of the control (100%) by that for 20 h. Appreciable carcinostatic activities are exhibited even the low doses of 10 and 25μM (36.0 and 18.5%, respectively). Moreover, hyperthermic treatment markedly enhanced the carcinostatic effect (23.0 and 4.3%, respectively). In measurement by GLC, DH16 : 0 was found in the cells [5], but the others showing low- or no activity were not (Fig. 3). The results suggest a close relation between their intracellular uptake and carcinostatic activity. Their penetrative effects through cell membranes is considered to be due to hydrophobicity or hydrophilicity by elongating or shortening the carbon side chain-length, whereas a lactone ring moiety is common to all DALs. An increase in molecular hydrophobicity may promote permeation of DALs through cell membranes, but this may be disadvantageous for intracellular uptake due to lower solubility in extracellular fluid. DH16 : 0 seems to have an appropriate hydrophobicity-hydrophilicity balance, in addition to the detergent-like activity, efficiently penetrates the cell membrane, and increases the intracellular concentration. The intracellular DALs may be converted to fatty compounds after the breakdown of lactone ring, producing reactive oxygen species (ROS), such as lipid peroxide (LPO) and superoxide anions [17], thus resulting in carcinostasis. Furthermore, hyperthermia is considered to increase intracellular ROS and induces cell injury [18], the temperature of cell membrane elevates, the membrane permeability promotes, the uptake of DHLs into the cells increases (see Fig. 3), and the production of cytotoxic substances enhances [19].

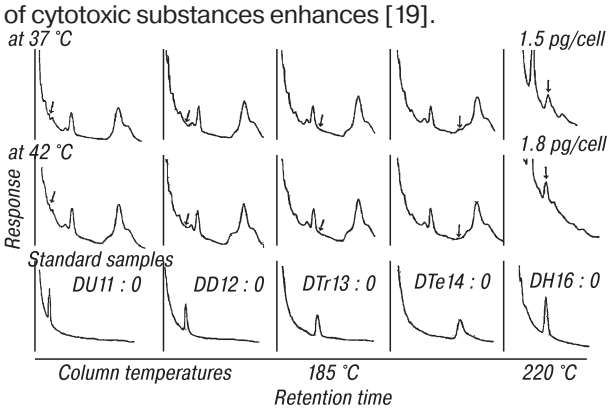


Fig. 3. Gas-liquid chromatograms of delta-alkyllactones incorporated into Ehrlich ascites tumor cells. Cells adjusted to a density of 2 x 10^5 cells/ml were incubated at a concentration of 25 μM in a humidified atmosphere of 5% CO_2 in air at 37 °C for 1 h. The suspension was then centrifuged, the supernatant was removed, and the packed cells were rinsed with MEM, and homogenized after removing the supernatant as much as possible and adding one ml of ethanol. The homogenate was poured into a glass tube sealed with cotton and the solvent of filtrates was evaporated. Ethanol was added to the residue (DAL) and the sample (3 μl) was analyzed with a gas chromatograph

As these results carcinostatic effect of DAIs on tumor cells enlarges. Among DALs DH16 : 0 exhibiting the highest carcinostatic activity against EAT cells has been clarified to be effective to lung NCI-H226 cells and stomach MKN74 cells by The Cancer Institute Foundation (Tokyo) which is attributed to The committee for New-Strategy-Based Antitumor-Agent Screening of The Japanese Ministry of Education, Culture, Sports, Science, and Technology [5].

Delta-hexadecanolactone (DH16 : 0), which has marked antitumor activity, is considered to be a potent antitumor regimen agent especially combined with hyperthermia.

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ПРОМОТОРНОЕ ДЕЙСТВИЕ ГИПЕРТЕРМИИ НА ЦИТОСТАТИЧЕСКУЮ АКТИВНОСТЬ ДЕЛЬТА-АЛКИЛЛАКТОНОВ В ОТНОШЕНИИ КЛЕТОК АСЦИТНОЙ ОПУХОЛИ ЭРЛИХА

Цель: **оценить промоторный эффект гипертермии на противоопухолевую активность новых низкомолекулярных (184–254 Да) дельта-алкиллактонов (DALs), химически синтезированных из разных макроциклических высокомолекулярных (348–439 Да) лактонов естественного происхождения, таких как камптотецин и салтриецин.** ***Методы:*** **суспензию клеток асцитной опухоли Эрлиха (EAT) смешивали с DAL в стеклянной пробирке, нагревали до 37 ˚C или 42 ˚C в течение 30 мин на водяной бане и далее культивировали при 37 ˚C в течение 20 или 72 ч. Оценку жизнеспособности клеток проводили с помощью WST-1 анализа, основанного на определении митохондриальной дегидрогеназы. Инкорпорированные в EAT-клетки DALs экстрагировали, их уровень измеряли с помощью газо-жидкостной хроматографии.** ***Результаты:*** **DALs значительно снижали жизнеспособность клеток после предварительной обработки при 42 ˚C по сравнению с 37 ˚C. При 37 ˚C были эффективными дельта-гексадекалактон (DH16 : 0) и дельта-тетрадекалактон (DTe14 : 0) (при 100 μM уровень выживаемости: 20,7; 66,1%; при 50 μM — 41,2; 82,4% соответственно). Этот эффект был более выраженным при 42 ˚C (при 100 μM 10,6; 27,6%; при 50 μM 30,6; 37,5% соответственно). Другие DALs, а именно дельта-ундекалактон (DU11 : 0), дельта-додекалактон (DD12 : 0) и дельта-тридекалактон (DTr13 : 0) были практически не эффективны. Оценка уровня выживаемости EAT-клеток, 30 мин обработанных DALs с последующим культивированием в течение 72 ч, показала повышенную канцеростатичсекую активность DH16 : 0 и DTe14 :0 даже при 25 μM концентрации, как при 37 ˚C (18,5; 78,5% соответсвенно), так и при 42 ˚C (5,0; 42,0% соответственно). Для других DALs данный эффект был незначительный либо отсутствовал. DH16 : 0 оставался эффективным как при 37 ˚C (36,0%), так и при 42 ˚C (23,0%) в 10 μM концентрации. В то же время только наиболее эффективный DAL — DH16 : 0 инкорпорировался в клетки EAT, и уровень инкорпорирования был выше при 42 ˚C, чем при 37 ˚C.** ***Выводы:*** **дельта-гексадеканолактон (DH16 : 0) показал наибольшую цитостатическую активность, которая значительно усиливалась в комбинации с гипертермией. Этот DAL можно рассматривать как потенциальный цитостатик, действие которого усиливается при гипертермии.**

Ключевые слова: **дельта-алкиллактон, дельта-гексадекалактон, гипертермия, противоопухолевая активность.**