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EFFECTS OF ETHYL-ESTERIZATION, CHAIN-LENGTHS, UNSATURATION DEGREES, AND HYPERTHERMIA ON CARCINOSTATIC EFFECT OF OMEGA-HYDROXYLATED FATTY ACIDS

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Aim: **To evaluate promotive effect of hyperthermia on the carcinostatic activity of synthesized omega-hydroxy fatty acids (ωHFAs) and their ethylesters agaist Ehrlich ascites tumor (EAT) cells.** ***Methods:*** **EAT cells were cultured with either ωHFAs or their ethylester derivatives in a water bath at either 37 ˚C or 42 ˚C for 30 min, followed by incubation in a CO_2 incubator for 20 or 72 h. Mitochondrial dehydrogenase-based WST-1 assay and trypan blue dye exclusion assay were then conducted after incubation. Morphological changes were observed by scanning electron microscopy (SEM).** ***Results:*** **Omega-HFA having a saturated 16-carbon straight-chain (ωH16:0) was the most carcinostatic (at 37 ˚C — viability level: 60.0%; at 42 ˚C — 49.6% (WST-1)) among saturated and unsaturated ωHFAs with 12, 15 or 16 carbon atoms, when administrated to EAT cells at 100 μM for 20 h. Carcinostatic activity was markedly enhanced by ethyl-esterization of saturated fatty acids, such as ωH16:0 (at 37 ˚C — 42.3%; at 42 ˚C — 11.2% ,** ***ibid*****) and ωH15:0 (at 37 ˚C — 74.6%; at 42 ˚C — 25.3% ,** ***ibid*****), and their unsaturated counterparts were extremely effective only in combination with hyperthermia. Prolongation of the incubation period to 72 h at the same concentration increased appreciably their carcinostatic effect (ωH16:0 ethylesther: 1.3%; ωH15:0 ethylesther: 8.0%). These values were also supported by dye exclusion assay. The carcinostatic activity enhanced more markedly by hyperthermia (1.2%; 2.1%,** ***ibid*****). SEM shows that ωH16:0 ethylester-exposed EAT cells underwent extensive injury, such as deformation of cell structure or disappearance of microvilli.** ***Conclusions:*** **ωH16:0 ethylester possesses high carcinostatic activity** ***in vitro*** **in combination with hyperthermia and may be utilized as potent anticancer therapeutic agent.**

Key Words: **antitumor activity, ω-hydroxy fatty acid, hyperthermia, WST-1 assay, scanning electronic microscopy.**

We have investigated the anti-tumor effects of fatty acids (R-COOH) [1, 2] and fatty alcohols (R-OH) [3, 4], and next those of hydroxyfatty acids (HFAs) (HO-R-COOH), whereas HFAs, such as 12-hydroxyeicosatetraenoic acid and 3-hydroxy analog, has been widely investigated their metabolism of [5–7], but their anti-tumor effects remain uncertain. Our results have shown that carcinostatic activity of free HFAs was low and increased by their esterifization [8]. In the present study, we took notice of hyperthermia which exhibits an anti-tumor effect on EAT cells [1, 9, 10] and enhancement of carcinostatic activity of ωHFAs was examined with combination by hyperthermia. The examination were systematically carried out using several methods under hyperthermia as follows: 1) carcinostatic effect of ωHFAs and their esters on EAT cells; 2) comparison of carcinostatic activities of ωHFAs and αHFAs; 3) effect of long-term exposure to ωHFAs; 4) morphological observation of treated cells by scanning electron microscope (SEM).

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Abbreviations used: DHA – docosahexaenoic acid; EAT – Ehrlich ascites tumor; ωH16:0 – 16-hydroxyhexadecanoic acid; ωHFAs – omega-hydroxy fatty acids; ωH15:0 – 15-hydroxypentadecanoic acid; WST-1 assay – mitochondrial dehydrogenase activity.

MATERIALS AND METHODS

Materials. All ω-HFAs were kindly provided by Soda Aromatic Co., Ltd. (Tokyo, Japan), and αHFAs were purchased from Sigma Chemical Co. (St. Louis, MO). They were dissolved in ethanol and stored in a freezer as test solutions. Table shows the ω-HFAs and derivatives examined in this study.

Table. Hydroxy fatty acids (ωHFA and αHFA) and derivatives used in the present study

Compound	Abbreviation	Purity
Free fatty acid		
12-Hydroxydodecanoic acid	ωH12:0	97.0%
15-Hydroxypentadecanoic acid	ωH15:0	99.0%
16-Hydroxyhexadecanoic acid	ωH16:0,	97.0%
2-Hydroxyhexadecanoic acid	αH16:0	98.0%
2-Hydroxyoctadecanoic acid	αH18:0	98.0%
2-Hydroxyeicosanoic acid	αH20:0	98.0%
15-Hydroxy-11-pentadecenoic acid	ωH15:1	92.7%
16-Hydroxy-9-hexadecenoic acid	ωH16:1	99.9%
Ester		
15-Hydroxypentadecanoic acid ethylester	ωH15:0 ethylester	99.8%
16-Hydroxyhexadecanoic acid ethylester	ωH16:0 ethylester	99.8%
15-Hydroxy-11-pentadecenoic acid ethylester	ωH15:1 ethylester	99.8%
16-Hydroxy-9-hexadecenoic acid ethylester	ωH16:1 ethylester	99.8%

Cells. Ehrlich ascites tumor (EAT) cells (RCB: No. 0142) obtained from female ICR mice with transplanted tumors were purchased from the Institute of Physical and Chemical Research (RIKEN BioResource Center, Cell Bank, Tsukuba, Japan). Cells were suspended in minimum essential medium (MEM) (GIBCO, Labs, Life Technolo-

gies, Inc., NY) supplemented with 10% fetal bovine serum (FBS) (GIBCO, Labs, Life Technologies, Inc., NY).

Cell culture, exposure of tumor cells to ωHFAs and hyperthermia. Cells were suspended in culture medium at a density of 2×10^5 (20 h cultures) or 2×10^4 (72 h culture) cells/mL. Aliquotes (μl) of the test solution were added to test tube. After the solvent was evaporated by a jet flow of nitrogen gas, culture medium was added to a residue (ωHFA), and the sample was sonicated. The cell suspension and test substance were mixed in a glass sample bottle (14 mm i. d. × 40 mm long). The cells were finally adjusted to a cell density of 1×10^5 or 1×10^4 cells/mL. The suspension in a tightly stopped tube was incubated in a water bath (Model BT-23, Yamato Scientific Co., Ltd., Tokyo, Japan) at 37 °C or 42 °C for 30 min. Bottles were covered with glass caps, and were then cultured in a humidified atmosphere of 5% CO_2 in air at 37 °C for 20 h or 72 h.

Cell viability assay. Cell viability was measured using two different methods: 1) The redox indicator dye WST-1 [11, 12] (Cell counting kit, Dojin Chemicals, Kumamoto, Japan) was used to detect the degree of mitochondrial dehydrogenase activity. The cultured cell suspension was transferred into a sampling tube and centrifuged. The resultant supernatant was removed from the tube, and 110 μL of WST-1 solution (8%) was added to the cell precipitate, which was then suspended and transferred into each of 96 wells of a microplate. After incubation at 37 °C for 1 h, diformazan formation was determined by absorption at 450 nm [11, 12] using a plate reader (Benchmark, Bio-Rad Laboratories, CA); 2) Dye-exclusion assay was performed after the cultured cells were treated as described above. Freshly prepared trypan blue solution in MEM (0.2%, 60 μL) was added to 60 μL of cell suspension, and counts of living (unstained) and dead (stained) cells were conducted under a microscope [13–15].

Morphological observation of cells incubated in the presence of ωHFA ethylesters in combination with hyperthermia. Cells were incubated in the presence of ωH16:0 ethylester at 37 °C or 42 °C for 20 h. They were then fixed with 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) at room temperature for 2 h. Specimens were then placed in 0.1 M phosphate buffer overnight, postfixed with 1% osmium tetroxide for 2 h, and then washed in re-distilled water (RDW), followed by dehydration through a graded series of ethanol. For scanning electron microscope (SEM) observation, samples were transferred to *tert*-butyl alcohol, and dried using a freeze-drier (ES-2030, Hitachi, Tokyo, Japan), sputter-coated with gold-palladium and examined under a Hitachi S-2460N SEM operated at 5 kV [16].

Statistical analysis. Experimental values are represented as means ± SD. Student's *t*-test was used to evaluate the significance of differences between groups, and differences were considered significant at $p < 0.05$.

RESULTS

Carcinostatic activity of ωHFAs. Carcinostatic effects on cells cultured for 20 h after the treatment at 37 °C or at 42 °C were measured using the mitochondrial dehydrogenase-based WST-1 assay (Fig. 1, *a*). The viability of experimental samples was evaluated by taking the viability of the untreated control group as 100%. Among saturated ωHFAs, only ωH16:0 significantly reduced cell survival rate (60.0%; n = 20, $p < 0.01$) at 37 °C. The other saturated and unsaturated fatty acids had no effect on cell survival: ωH12:0 (96.0%); ωH15:0 (97.9%); ωH16:1 (95.4%); and ωH15:1 (99.0%). On the other hand, hyperthermia at 42 °C reduced viability (cell survival rate) to 64.0% (n = 20) when compared to the control group (37 °C). Cell survival was further reduced with ωH16:0 and hyperthermia (49.6%; n = 20, $p < 0.01$), and thus the carcinostasis of these agents was more marked than that of hyperthermia alone. The other fatty acids were ineffective; ωH12:0 (n = 12, 59.9%), ωH16:1 (n = 12, 54.9%), ωH15:0 (n = 12, 64.4%), and ωH15:1 (n = 12, 71.4%).

The results of the Trypan blue dye-exclusion assay (Fig. 1, *b*) were nearly consistent with those of WST-1 assay. Viability of EAT cells decreased to 49.8% (n = 6, $p < 0.01$) in ωH16:0 at a dose of 100 μM as compared with the control, whereas ωH12:0 (n = 6, 92.5%), ωH15:0 (n = 6, 80.5%), ωH15:1 (n = 6, 84.1%) and ωH16:1 (n = 6, 96.8%) were scarcely carcinostatic at the same dose at 37 °C. In addition, ωH16:0 was the only drug that was carcinostatic at both 37 °C and 42 °C, while the other ωHFAs were ineffective.

Of the ωHFAs examined, ωH16:0 was the most carcinostatic and had the largest number of carbon atoms. This suggests that ωH18:0 or ωH20:0 may be more carcinostatic than ωH16:0; however, we were unable to obtain these ωHFAs. Therefore, we understood that carcinostatic intensity between ωHFAs and αHFAs was same, if the activity of ωH16:0 was almost equal to that of αH16:0. The carcinostatic activity of αH16:0, αH18:0 and αH20:0 purchased instead of ωH16:0, ωH18:0 and ωH20:0, respectively, was compared with that of ωH16:0 (Fig. 2). At 37 °C, αH16:0 (n = 6, 57.2%, $p < 0.01$) and αH18:0 (n = 6, 61.6%, $p < 0.01$) exhibited carcinostatic activity similar to that (n = 6, 59.2%, $p < 0.01$) of ωH16:0 at 100 μM, but αH20:0 (n = 6, 85.5%) was less carcinostatic than ωH16:0. At 42 °C, cell viability was reduced to 62.4% in the absence of HFA and to 29.3% (n = 6, $p < 0.01$) in the presence of ωH16:0. Carcinostatic activity of αH16:0 and αH18:0 was nearly equal to that of ωH16:0, while αH20:0 exhibited lower activity.

Enhancement of carcinostatic activity of ωHFA ethylesters. The carcinostatic effects of ωHFA ethylesters on EAT cells (cultured for 20 h) were assessed by WST-1 and trypan blue exclusion assay (Fig. 3, *a* and *b*) in the same way as for free ωHFAs. At 37 °C, carcinostasis was markedly enhanced by the ωHFA ethylester derivatives of saturated fatty chains; ωH16:0 ethylester was the most potent, with cell viability decreasing to 42.3% on WST-1 assay (n = 12, $p < 0.001$) and 46.7% on Trypan blue dye-exclusion assay (n = 6, $p < 0.001$) at 50 μM. Carcinostatic activity was followed in order by ωH15:0 ethylester (74.6% and 63.2%, respectively), ωH16:1 ethylester (93.7% and 81.9%, respectively) and ωH15:1 ethylester (93.5% and 96.8%, respectively). At 100 μM, ωH16:0 ethylester substantially diminished cell viability to 1.1% and 0.8% on

WST-1 and Trypan blue dye-exclusion assays, respectively ($p < 0.001$), followed by ωH15:0 ethylester (2.7% and 1.2%, respectively; $p < 0.001$), ωH16:1 ethylester (55.6% and 54.4%%, respectively) and ωH15:1 ethylester (81.0% and 85.4%, respectively).

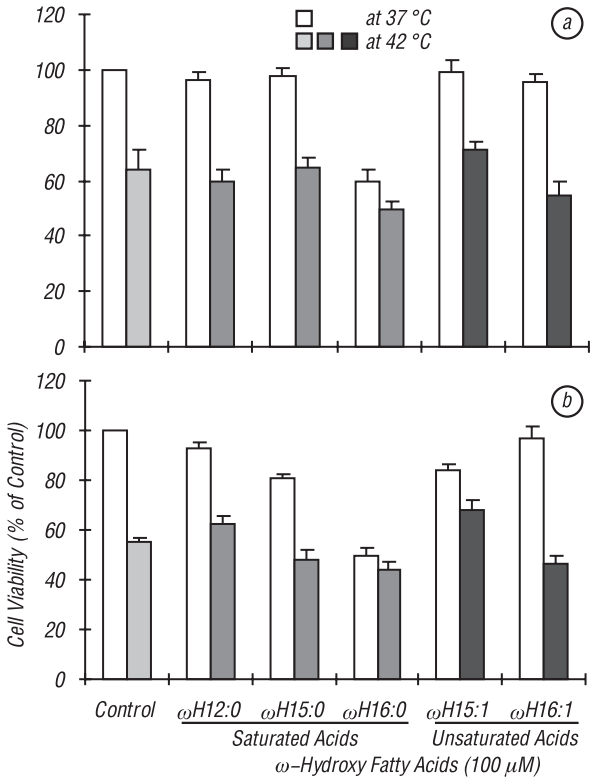

Fig. 1. Cytotoxic effects of omega-hydroxyl fatty acids (ωHFAs) on Ehrlich ascites tumor (EAT) cells, as measured by mitochondrial dehydrogenase-based WST-1 assay. Cells were seeded at a density of 1×10^5 cells/mL, incubated in the presence of each ωHFA at a dose of 100 μM at 37 °C or 42 °C for 30 min and maintained by sequential culture at 37 °C for 20 h. *a:* Cell viability as measured by the WST-1 assay. *b:* Cytotoxic effects of ωHFAs on EAT cells as measured by Trypan blue dye-exclusion assay (cells were treated as for Fig. 1, *a*). Unstained and stained cells in the presence of Trypan blue were counted as viable cells and dead cells, respectively, under an optical microscope
Note: Data are means (n = 20–6); bars indicate S.D.; *$p < 0.01$.

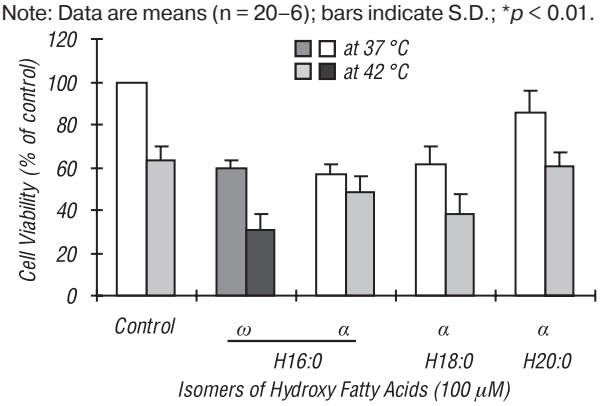

Fig. 2. Cytotoxic effects of hydroxyl fatty acid isomers (ωHFAs and αHFAs) on Ehrlich ascites tumor cells as measured by WST-1 assay. Cells were treated as described for Fig. 1, *a*
Note: Data are means (n = 6); bars indicate S.D.; *$p < 0.01$.

Hyperthermia at 42 °C enhanced carcinostasis, and at 100 μM, even ωH16:1 and ωH15:1 ethylesters diminished viability to 4.5% (n = 12, $p < 0.001$) and 12.9% (n = 12, $p < 0.001$), respectively (Fig. 3, *a*). Fig. 3, *b* shows the cytotoxic effects of ωHFA ethylesters on tumor cells, as assessed by trypan blue assay. At 50 μM, cytotoxic activity with ωH16:0 ethylester decreased (11.2%, n = 12, $p < 0.001$), ωH15:0 ethylester (25.3%, n = 12, $p < 0.01$), ωH15:1 ethylester (52.7%, ns) and ωH16:1 ethylester (41.5%, ns). In addition, a majority of the observed EAT cells exhibited fragmentation or cytolysis after incubation with ωH16:0 ethylester (0.2%, $p < 0.001$) or ωH15:0 ethylester (0.6%, $p < 0.001$) at 100 μM.

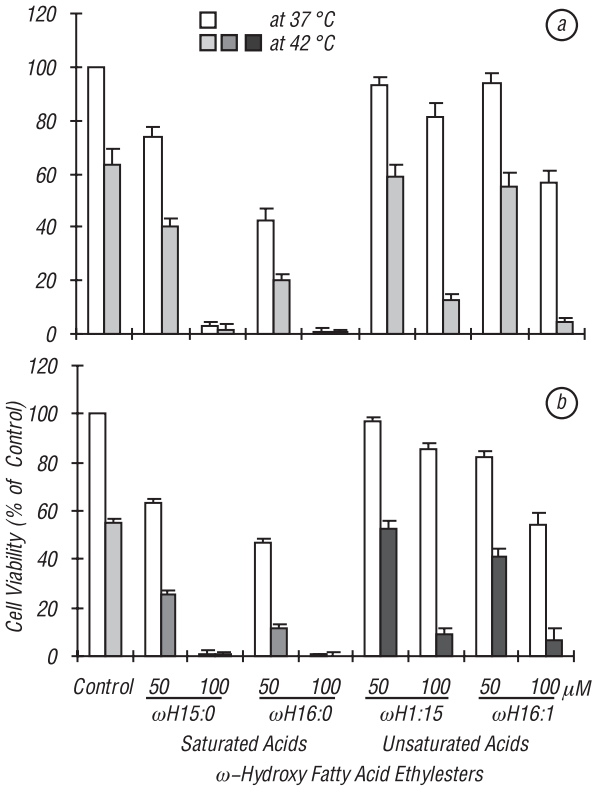

Fig. 3. *a:* Cytotoxic effects of ωHFA ethylesters at 50 or 100 μM on EAT cells as measured by WST-1 assay. Cells were seeded at a density of 1×10^5 cells/mL, incubated in the presence or absence of ωHFA ethylester at 37 °C or 42 °C for 30 min and cultured at 37 °C for 20 h. *b:* Cytotoxic effects of ωHFA ethylesters at 50 or 100 μM on tumor cells at 37 °C or 42 °C as measured by Trypan blue exclusion assay (cells were treated as for Fig. 2, *a*)
Note: Data are means (n = 12 and 6, respectively); bars indicate S.D.; *$p < 0.01$; **$p < 0.001$.

To examine the effect of long-term exposure to markedly effective ωH16:0 and ωH15:0 ethylesters, the cells were further cultured at 37 °C for 72 h and subjected to WST-1 (Fig. 4, *a*) and Trypan blue assays (Fig. 4, *b*), and the results showed good agreement. Cell viability decreased markedly to 59.1% and 74.4% ($p < 0.001$), respectively, with ωH16:0 ethylester at a dose of 25 μM, and in combination with hyperthermic treatment, viability decreased to 30.2% and 18.2 ($p < 0.001$), respectively. Addition of ωH15:0 ethylester at 25 μM resulted in small effectiveness either at 37 °C or 42 °C. At 50 μM, they exhibited nearly perfect diminution of cell viability either at 37 °C or 42 °C.

After hyperthermia at 42 °C, extensive damage to the cell surface was observed, as shown in Fig. 5, in contrast to control cells with normal microvilli.

Furthermore, marked cell destruction and fragmentation occurred when hyperthermia was combined with ωH16:0 ethylester. There were no morphological differences in control cells at 37 °C and 42 °C (Fig. 5, *a, d*).

Extensive damage to cells was observed at 37 °C with ωH16:0 ethylester (Fig. 5, *b, c*), and this damage was markedly accentuated when hyperthermia was combined with ωH16:0 ethylester (Fig. 5, *e, f*).

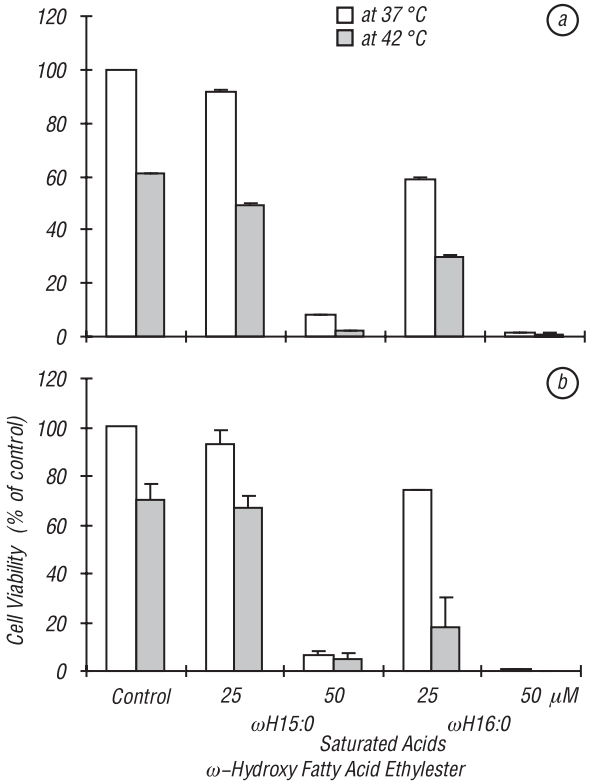

Fig. 4. Cells were seeded at a density of 1×10^4 cells/mL, incubated in the presence or absence of ωHFA ethylester at 37 °C or 42 °C for 30 min and cultured at 37 °C for 72 h. Cytotoxic effects were measured by *a:* WST-1 assay, and *b:* Trypan blue dye-exclusion assay

Note: Data are means (n = 12 and 6, respectively); bars indicate S.D.; $*p < 0.01$; $**p < 0.001$.

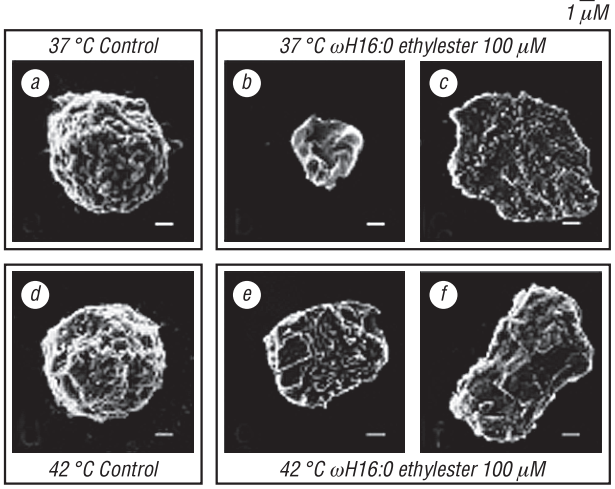

Fig. 5. Scanning electron micrographs of Ehrlich ascites tumor cells exposed to ωHFA ethylesters. Cells were incubated in the presence of ωH16:0 ethylester at a dose of 100 μM at 37 °C or 42 °C for 30 min, cultured at 37 °C for 20 h, and were then conventionally fixed and washed. Cells were again fixed with 1% osmic acid, washed and dehydrated. Cells were coated with ions after lyophilization, and cell shape was observed by SEM (× 6.0 K)

DISCUSSION

In the present study, the carcinostatic effects of ω-hydroxyfatty acids (ωHFA)s and their ethylesters were evaluated by assays for mitochondrial dehydrogenase activity and dye exclusion. The results revealed that of free acids, H16:0 exhibited the highest carcinostatic activity and of all free acids and their ethylesters, H16:0 ethylester had the most potent carcinostatic action. Their carcinostatic effects were markedly enhanced with elongating the cell culture period. The ethylester at 50 μM almost perfectly diminishes the cell viability by the exposure for 72 h, whereas the survial rate is 43.2% in the culture for 20 h, and the carcinostatic activity is exhibited even the low dose of 25 μM (Fig. 4). Moreover, hyperthermia enhances markedly those effects.

SEM revealed extensive cellular destruction, such as the disappearance of cell-surface microvilli and deformed shape, in EAT cells incubated with ωH16:0 ethylester (Fig. 5). Thus, the cytotoxic activity of hydroxyfatty acid compounds may be attributed to either their surface-denaturing activity on the cell membrane or their destruction of cellular organelles after intracellular uptake [5]. 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 [5] and fatty alcohols [7].

Although ωH16:0 having the largest number of carbon atoms of the non-esterified compounds examined was the most carcinostatic, its activity might be lower than that of ωH18:0 or ωH20:0. With the examination using αHFAs, our results suggest that the activities of αH16:0 and αH18:0 was nearly equal to that of ωH16:0 at either 37 °C or 42 °C but αH20:0 was scarcely carcinoststic. In measurement by GLC, H16:0 and its ethylester were found in the cells, but the others showing low- or no activity were not [8]. 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. An increase in molecular hydrophobicity may promote permeation of HFAs through cell membranes, but this may be disadvantageous for intracellular uptake due to lower solubility in extracellular fluid [4]. H16:0 and its ethylesther seem to have an appropriate hydrophobicity-hydrophilicity balance, in addition to the detergent-like activity, efficiently penetrates the cell membrane, and increases the intracellular concentration, producing cytotoxic substances, such as hydrogen peroxide and superoxide anions [17, 18], thus resulting in carcinostasis.

Thus, administration of hydroxyhexadecanoic acid (H16:0) ethylester in combination with hyperthermia could be considered as an attractive mean for treatment of cancer.

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ВЛИЯНИЕ ЭТИЛЭТЕРИФИКАЦИИ, ДЛИНЫ ЦЕПИ, СТЕПЕНИ НЕНАСЫЩЕННОСТИ И ГИПЕРТЕРМИИ НА КАНЦЕРОСТАТИЧЕСКОЕ ДЕЙСТВИЕ ОМЕГА-ГИДРОКСИЛИРОВАННЫХ ЖИРНЫХ КИСЛОТ

Цель: **проанализировать усиливающий эффект гипертермии на канцеростатическую активность синтезированных омега-гидроксилированных жирных кислот (ωHFAs) и их этиловых эфиров по отношению к клеткам асцитной опухоли Эрлиха (EAT).** ***Методы:*** **клетки EAT инкубировали с ωHFAs или их этилэфирными производными на водяной бане при 37 °C или 42 °C в течение 30 мин с дальнейшим культивированием в CO_2 инкубаторе на протяжении 20 или 72 ч, после чего анализировали жизнеспособность клеток методами анализа WST-1, основанного на активности митохондриальных дегидрогеназ, и по включению трипанового синего. Морфологические изменения клеток определяли с использованием сканирующей электронной микроскопии.** ***Результаты:*** **при культивации клеток EAT в присутствии 100 μM соединений в течение 20 ч омега-HFA с насыщенной 16-углеродной прямой цепью (ωH16:0) проявляли наиболее выраженный канцеростатический эффект (при 37 °C уровень жизнеспособности составил 60,0%; при 42 °C — 49,6% (WST-1)) по сравнению с таковым насыщенных и ненасыщенных ωHFAs, содержащих 12, 15 или 16 атомов углерода. Канцеростатическая активность значительно возрастала при этилэтерификации насыщенных жирных кислот, таких как ωH16:0 (при 37 °C — 42,3%; при 42 °C — 11,2%, *ibid*) и ωH15:0 (при 37 °C — 74,6%; при 42 °C — 25,3% , *ibid*), в то время как производные ненасыщенных кислот были высокоэффективны только в комбинации с гипертермией. Увеличение периода инкубации клеток до 72 ч при той же концентрации веществ приводило к значительному увеличению их канцеростатического действия (этиловый эфир ωH16:0 — 1,3%; этиловый эфир ωH15:0 ethylesther — 8,0%), подтвержденного данными окраски трипановым синим. Применение гипертермии также усиливало канцеростатическое действие соединений (1,2%; 2,1%, *ibid*). Результаты исследования методом SEM показали, что клетки EAT, инкубированные с этиловым эфиром ωH16:0, разрушаются с нарушением клеточной структуры и исчезновением микроволокон.** ***Выводы:*** **в комбинации с гипертермией этиловый эфир ωH16:0 проявляет высокую канцеростатическую активность *in vitro*, что говорит о возможности применения соединения в терапии опухолевых заболеваний.**

Ключевые слова: **противоопухолевая активность, ω-гидроксилированные жирные кислоты, гипертермия, анализ WST-1, сканирующая электронная микроскопия.**