

# The antitumor activities of the structurally-similar two-species aromatics Tonalide and Pearlide and the enhancement of their effects by hyperthermia

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Received July 18, 2008; Accepted September 25, 2008

DOI: 10.3892/mmr\_00000058

**Abstract.** Antitumor activities have been reported for the aromatics Tonalide (6-acetyl-1,1,2,4,4,7-hexamethyl-tetrahydronaphthalene, AHTN) and Pearlide (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta- $\gamma$ -2-benzopyran, HHCB), which are contained in detergents. In this study, their carcinostatic activities in Ehrlich ascites tumor (EAT) cells were evaluated by mitochondrial dehydrogenase-based WST-1 assay and dye-exclusion assay. The viability of EAT cells treated at 37 or 42°C for 30 min and sequentially cultured at 37°C was assayed at graded times. Immediately after treatment at 37°C, neither Tonalide nor Pearlide had an effect on EAT cells, even at a concentration as high as 200  $\mu$ M. However, cell viability was reduced to 40% versus the control after 20 h of culture with Tonalide at 50  $\mu$ M, and to below 20% at 25  $\mu$ M after 72 h. In contrast, Pearlide was nearly inactive, even at a dose of 100  $\mu$ M after 20 h of culture, and only reduced cell viability to 41.2% after 72 h. After treatment at 42°C without culture, neither of the aromatics was effective, even at a dose of 200  $\mu$ M. The viability of cells cultured with Tonalide for 20 h after treatment at 42°C was reduced to nearly half of that at 37°C, and to 10% of the control after culture for 72 h. These values for the reduction of cell viability were also achieved by the Trypan blue dye-exclusion assay. The lifespan-prolonging effects of Tonalide on mice implanted with EAT cells were also examined. The lifespan of mice administered 1.8 mg/day of Tonalide was prolonged by up to 28 days, while mice that did not receive Tonalide died within a mean of 15 days. Thus, Tonalide exhibited marked carcinostatic effects *in vitro* and *in vivo* and may prove to be a potent multipurpose antitumor agent in combination with hyperthermia.

## Introduction

The antitumor activities of aromatics, such as natural musks, have been reported (1-3). We studied the carcinostatic activity of diverse aromatics in a series screening anticancer agents (4,5) and found Tonalide (6-acetyl-1,1,2,4,4,7-hexamethyl-tetrahydronaphthalene, AHTN), an aromatic widely used in some detergents or soaps, to have carcinostatic activities. The antitumor effects of tonalide remain to be elucidated, whereas its toxicity has been reported in environmental (6-8) and biological (9-11) studies.

In the present study, the antitumor effects of Tonalide and a similar aromatic, Pearlide (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta- $\gamma$ -2-benzopyran, HHCB), were examined in Ehrlich ascites tumor (EAT) cells. Synergistic enhancement of the effects of Tonalide and Pearlide was observed by applying them in combination with hyperthermia, which is known to inhibit the growth (12-14) and DNA synthesis (15-17) of tumor cells. Carcinostatic effect was evaluated as a decrease in mitochondrial dehydrogenase activity reflecting the survival rate of cells as measured by the WST-1 assay (18,19), and also as a decrease in viable cells according to the degree of staining determined by the trypan blue dye-exclusion assay (20,21). Lifespan-prolonging effects on EAT cell-transplanted mice were investigated to evaluate whether the substances could be safely administered to cancer patients as anticancer drugs (4).

## Materials and methods

**Materials.** Tonalide (AHTN) and Pearlide (HHCB) were kindly provided by Soda Aromatic Co. Ltd. (Tokyo), dissolved in ethanol and stored in a freezer as test solutions. Details are shown in Table I.

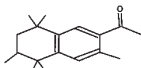
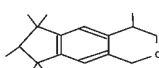
**Cells.** Ehrlich ascites tumor cells (RCB: No. 0142) were purchased from the Institute of Physical and Chemical Research (Tsukuba, Japan). The cells were cultured in Eagle's minimum essential medium (MEM) (Gibco Labs Life Technologies Inc., NY) supplemented with 10% fetal bovine serum (Gibco Labs Life Technologies Inc.).

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**Key words:** Tonalide, Pearlide, antitumor activity, hyperthermia

Table I. Characteristics and chemical structure of Tonalide and Pearlide.

Brand name	Tonalide	Pearlide
Chemical name	6-acetyl-1,1,2,4,4,7-hexamethyl-1,2,3,4-tetrahydronaphthalene	1,3,4,6,7,8,-hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta-γ-2-benzopyran
Abbreviation	AHTN	HHCB
Chemical formula	C <sub>18</sub> H <sub>26</sub> O	C <sub>18</sub> H <sub>26</sub> O
Molecular weight	258	258
Purity (GC%)	98.0	99.1
Boiling point	248°C/101.3 kPa	129°C/0.11 kPa
Flash point	53°C	157°C
Solubility	Soluble in ethanol; insoluble in water	Soluble in ethanol; insoluble in water
Chemical structure		

**Examination for carcinostatic effects in cultured tumor cells.** Examination was essentially conducted as previously described (5,17). In brief, cells were suspended in culture medium at a density of  $2 \times 10^5$  or  $2 \times 10^4$  cells/ml for a 20- or 72-h culture test, respectively. An aliquot of the test solution was placed in a test tube. After the solvent was evaporated by a jet flow of nitrogen gas, culture medium was added to the Tonalide or Pearlide residue and sonicated. The cell suspensions and the contents of the test tube were combined in a glass sample bottle (14 mm i.d. x 40 mm) and the cells were diluted with adjustment to a cell density of  $1 \times 10^5$  or  $1 \times 10^4$  cells/ml. Finally, 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<sub>2</sub> at 37°C for 20 or 72 h.

**Cell viability assay.** The viability of cells was measured using two different methods: i) the redox indicator dye WST-1, which becomes more chromic according to mitochondrial dehydrogenase activity, and ii) the trypan blue dye-exclusion assay. For WST-1 (Cell Counting Kit, Dojin Chemicals, Kumamoto, Japan) (18,19), the cultured cell suspension was transferred to a sampling tube and centrifuged. The resultant supernatant was completely removed from the tube and 110  $\mu$ l of WST-1 (8%) per well was added to the cell precipitate, suspended and transferred to each well of a 96-well microplate. After incubation at 37°C for 1 h, the resultant diformazan was determined by measuring the absorption at 450 nm with a plate reader (Benchmark, Bio-Rad Laboratories, CA). For the dye-exclusion assay (20), the cultured cell suspension was treated as above, then 60  $\mu$ l of freshly-prepared trypan blue dye solution in MEM (0.20%) was added to 60  $\mu$ l of cell suspension,

and microscopic counts of living (unstained) and dead (stained) cells were conducted on a hemocytometer (21).

**Lifespan of tumor-implanted mice injected with Tonalide.** ddY mice (Shimizu Experimental Materials, Kyoto, Japan) were intraperitoneally implanted with EAT cells (RCB: No. 0142), divided into three groups (8-10 mice per group) and treated once daily for five consecutive days with 0.6, 1.2 or 1.8 mg of Tonalide, respectively. The lifespans were recorded. Tonalide was selected for having shown marked carcinostatic activity *in vitro*.

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

## Results

**Carcinostatic effects of Tonalide and Pearlide immediately following hyperthermic treatment.** Tonalide or Pearlide was added to EAT cells and the samples were heated in a water bath at 37 or 42°C for 30 min. The carcinostatic effects of the substances were immediately measured using the WST-1 assay (Fig. 1). The cell viability of the control in the absence of either drug was set as 100% and compared to the viability of the experimental samples at graded doses. Though hyperthermia alone reduced cell viability to 80% of the control, a combined effect was not observed with either aromatic. Indeed, Tonalide and Pearlide scarcely exhibited carcinostatic effects, even at a high concentration of 200  $\mu$ M. In contrast, Tonalide at 100 or 200  $\mu$ M has a tendency to increase cell viability.

**Carcinostatic effects of Tonalide and Pearlide on short-term (20-h) or long-term (72-h) cultures.** Tonalide or Pearlide was added to EAT cells and the samples were heated in a water bath at 37°C for 30 min, then maintained by sequential culture

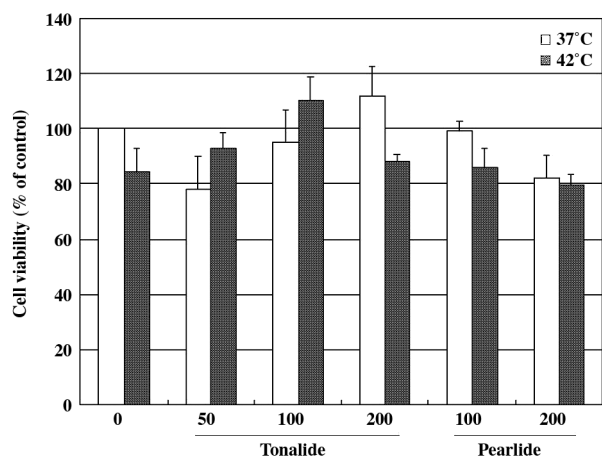


Figure 1. Cytotoxic effects of Tonalide and Pearlide on Ehrlich ascites tumor cells treated at 37 or 42°C without sequential culture. Cells were seeded at a density of  $1 \times 10^5$  cells/ml, incubated in the presence or absence of Tonalide or Pearlide at graded doses of 50–200  $\mu$ M at 37 or 42°C for 30 min. Viability of the cells was immediately measured at an absorption of 450 nm using the WST-1 assay. The absorbance of cells in the absence of Tonalide and Pearlide at 37°C was  $0.822 \pm 0.062$  (the control value). Data shown represent the means  $\pm$  SEM of quadruplicate measurements as percentages of the control value.

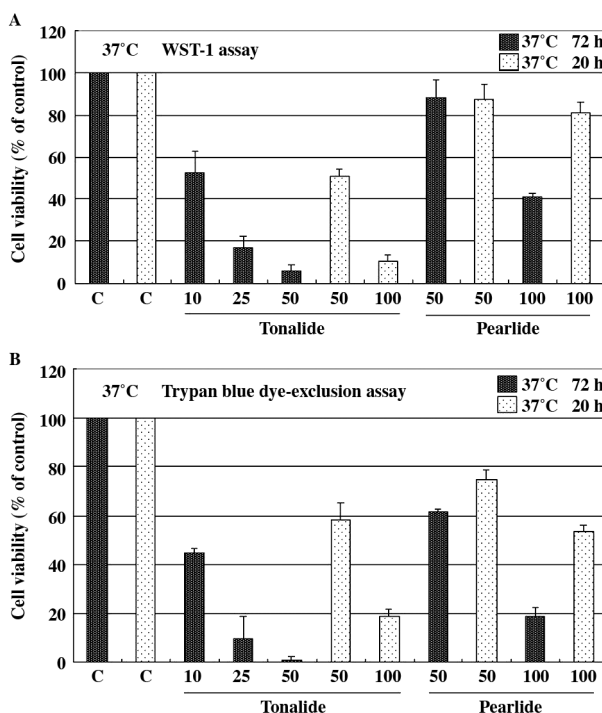


Figure 2. (A) Short- and long-term carcinostatic effects of Tonalide and Pearlide on Ehrlich ascites tumor (EAT) cells as measured by mitochondrial dehydrogenase-based WST-1 assay. Cells were seeded, incubated in the presence or absence of graded doses of Tonalide or Pearlide at 37°C for 30 min, and further maintained by sequential culture at 37°C for 20 or 72 h. Viability of the cells was measured using the WST-1 assay. The absorbance of cells treated for 20 and 72 h in the absence of Tonalide and Pearlide at 37°C was  $1.778 \pm 0.383$  or  $1.445 \pm 0.118$  (the control values), respectively. Data shown represent the means  $\pm$  SEM of quadruplicate measurements as percentages of the control value. (B) Short- and long-term carcinostatic effects of Tonalide and Pearlide on EAT cells as measured by trypan blue dye-exclusion assay. Cells were treated as described in (A). Viability of the cells was evaluated using the trypan blue dye-exclusion assay. The number of viable cells in the absence of Tonalide and Pearlide at 37°C was  $(1.70 \pm 0.70) \times 10^5$  or  $(1.00 \pm 0.10) \times 10^5$  (the control values). Experimental values represent the means  $\pm$  SEM of triplicate measurements as percentages of the control value.

in a humidified atmosphere of 5%  $\text{CO}_2$  at 37°C for 20 or 72 h. The carcinostatic effects of the substances were measured using the WST-1 assay (Fig. 2A). In the 20-h culture, the viability of the experimental samples at a dose of 50 or 100  $\mu$ M was evaluated. Only Tonalide was carcinostatic, with a cell survival rate of  $50.6 \pm 3.4\%$  ( $p < 0.0001$ ) at 50  $\mu$ M and  $10.4 \pm 3.1\%$  ( $p < 0.0001$ ) at 100  $\mu$ M. Pearlide was almost ineffective, even at 100  $\mu$ M.

To examine the effect of long-term exposure on each aromatic, the cells were further cultured at 37°C for 72 h and carcinostatic activity was measured using the WST-1 assay (Fig. 2A). At a dose of 10, 25 or 50  $\mu$ M, Tonalide reduced cell viability to  $52.7 \pm 10.0\%$  ( $p < 0.0001$ ),  $16.8 \pm 5.5\%$  ( $p < 0.0001$ ) and  $5.8 \pm 2.6\%$  ( $p < 0.0001$ ), respectively. The values at 25  $\mu$ M correspond to those at 50  $\mu$ M in the 20-h culture, and the values at 50  $\mu$ M are 2-fold those at 100  $\mu$ M. Pearlide, in the 72-h culture, decreased viability to  $41.2 \pm 14.8\%$  ( $p < 0.0001$ ) at 100  $\mu$ M (and was almost inactive in the 20-h culture).

The carcinostatic activities of Tonalide and Pearlide in cells treated as described above were also measured using the trypan blue dye-exclusion assay (Fig. 2B). Tonalide at doses of 50 and 100  $\mu$ M reduced cell viability to  $58.2 \pm 6.9\%$  ( $p < 0.0001$ ) and  $18.7 \pm 2.9\%$  ( $p < 0.0001$ ) respectively, versus that of the control, whereas Pearlide was not markedly carcinostatic ( $74.7 \pm 3.9\%$  and  $53.6 \pm 2.4\%$ ).

The viability of the cells cultured for 72 h after treatment at 37°C was decreased by Tonalide to  $44.5 \pm 2.0\%$  ( $p < 0.0001$ ) at 10  $\mu$ M,  $9.6 \pm 8.8\%$  ( $p < 0.0001$ ) at 25  $\mu$ M, and thoroughly at 50  $\mu$ M.

The values at 25  $\mu$ M correspond to those at 50  $\mu$ M in the 20-h culture, and the values at 50  $\mu$ M are 2-fold those at 100  $\mu$ M. Pearlide was not effective at 50  $\mu$ M for 72 or 20 h, but reduced viability to  $18.5 \pm 3.6\%$  ( $p < 0.0001$ ) at 100  $\mu$ M. The results obtained using the dye-exclusion assay roughly coincide with those of the WST-1 assay.

*Effect of hyperthermia on carcinostatic effects of Tonalide and Pearlide.* The viability of EAT cells cultured with the aromatics for 20 or 72 h after treatment at 42°C for 30 min was measured using the WST-1 assay (Fig. 3A). Hyperthermia alone at 42°C reduced viability to  $65.0 \pm 5.9\%$  ( $p < 0.0001$ ) versus that of the control at 37°C, while Tonalide at doses of 50 and 100  $\mu$ M reduced viability to  $23.3 \pm 2.6\%$  ( $p < 0.0001$ ) and  $2.7 \pm 1.0\%$  ( $p < 0.0001$ ) respectively, indicating that hyperthermia had a marked enhancing effect. At 42°C, cell viability with Pearlide at the same doses, which were almost ineffective at 37°C, also decreased to  $48.3 \pm 1.0\%$  ( $p < 0.0001$ ) and  $30.7 \pm 4.7\%$  ( $p < 0.0001$ ), respectively.

In the long-term 72-h culture after hyperthermic treatment alone at 42°C, cell viability was reduced to  $61.2 \pm 5.8\%$  ( $p < 0.0001$ ) versus the control value at 37°C. At 42°C, Tonalide at doses of 10, 25 and 50  $\mu$ M reduced viability to  $39.4 \pm 2.1\%$  ( $p < 0.0001$ ),  $4.6 \pm 2.2\%$  ( $p < 0.0001$ ) and  $3.0 \pm 1.2\%$  ( $p < 0.0001$ ) respectively, indicating that hyperthermia had marked effects. Furthermore, though Pearlide at 50 and 100  $\mu$ M exhibited no effect at 37°C, at the same doses in combination with hyperthermia it reduced viability to  $13.1 \pm 2.0\%$  ( $p < 0.0001$ ) and  $5.1 \pm 2.4\%$  ( $p < 0.0001$ ), respectively.

The carcinostatic effects of Tonalide and Pearlide on cells cultured for 20 or 72 h after hyperthermia at 42°C were

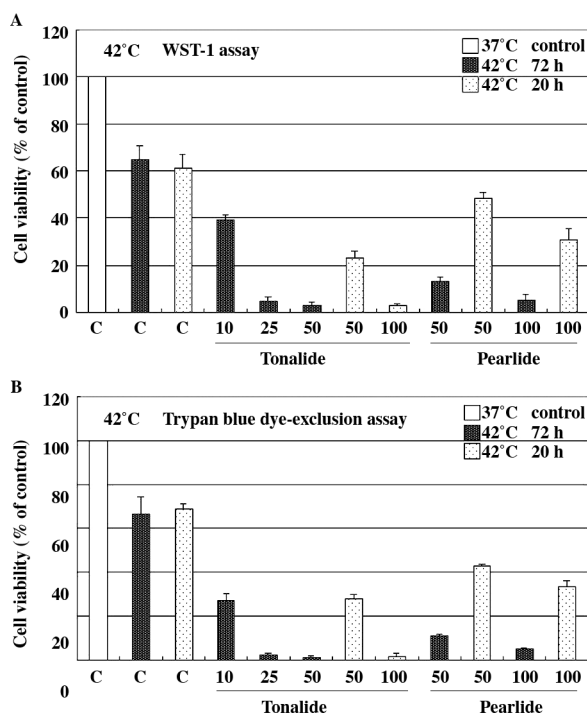


Figure 3. (A) Short- and long-term carcinostatic effects of Tonalide and Pearlide on Ehrlich ascites tumor (EAT) cells in combination with hyperthermia (as measured using the WST-1 assay). Cells were seeded and incubated in the presence or absence of Tonalide or Pearlide at graded doses at 42°C for 30 min, and further maintained by sequential culture at 37°C for 20 or 72 h. Viability of the cells was measured using the WST-1 assay. The absorbance of cells treated in the absence of Tonalide and Pearlide at 37°C for 20 or 72 h was  $1.778 \pm 0.383$  or  $1.445 \pm 0.118$  (the control values), respectively. Data represent the means  $\pm$  SEM of quadruplicate measurements as percentages of the control value. (B) Short- and long-term carcinostatic effects of Tonalide and Pearlide on EAT cells in combination with hyperthermia (as measured by trypan blue dye-exclusion assay). Cells were treated as described in Fig. 2. Viability of the cells was evaluated using the trypan blue dye-exclusion assay. The number and rate of viable cells in the absence of Tonalide and Pearlide at 37°C were  $(1.70 \pm 0.70) \times 10^5$  or  $(1.00 \pm 0.10) \times 10^5$  (the control values). Experimental values represent the means  $\pm$  SEM of triplicate measurements as percentages of the control value.

measured by trypan blue dye-exclusion assay (Fig. 3B). Hyperthermia alone at 42°C (20-h sequential culture) reduced viability to  $66.5 \pm 7.8\%$  ( $p < 0.0001$ ) versus the control at 37°C. Tonalide at doses of 50 and 100  $\mu\text{M}$  enhanced the reduction of cell viability to  $28.1 \pm 1.9\%$  ( $p < 0.0001$ ) and  $1.3 \pm 1.9\%$  ( $p < 0.0001$ ) respectively, versus hyperthermia alone (100%). Pearlide at the same doses reduced viability to  $42.6 \pm 1.0\%$  ( $p < 0.0001$ ) and  $33.5 \pm 2.4\%$  ( $p < 0.0001$ ), respectively.

After 72 h of culture following hyperthermia alone at 42°C, cell viability was reduced to  $68.9 \pm 2.4\%$  ( $p < 0.0001$ ) of the control. Tonalide at 10 and 25  $\mu\text{M}$  reduced viability to  $27.3 \pm 2.8\%$  ( $p < 0.0001$ ) and  $2.4 \pm 0.9\%$  ( $p < 0.0001$ ), respectively; at the latter dose, cells almost died. Pearlide at 50 and 100  $\mu\text{M}$  reduced viability to  $10.5 \pm 1.3\%$  ( $p < 0.0001$ ) and  $5.1 \pm 0.4\%$  ( $p < 0.0001$ ), indicating that combination with hyperthermia had an enhancing effect. These results were substantially consistent with those of the WST-1 assay.

*Lifespan of tumor-implanted mice treated with Tonalide.* The lifespan-prolonging effects of Tonalide, which has marked

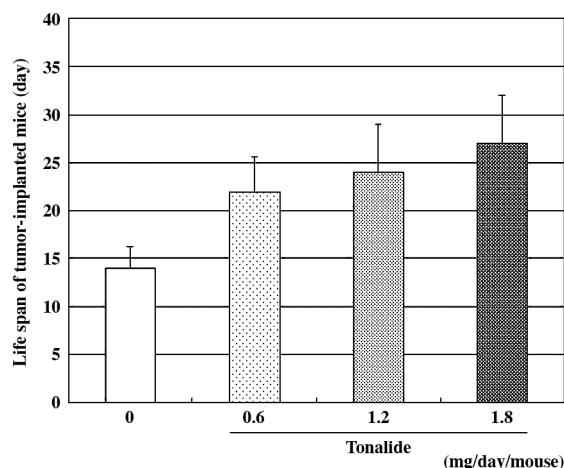


Figure 4. Lifespan-prolonging effects of Tonalide on Ehrlich ascites tumor (EAT) cell-transplanted mice. ddY mice were intraperitoneally transplanted with EAT cells and treated intraperitoneally with Tonalide at a dose of 0.6, 1.2 or 1.8 mg/day for five consecutive days. Data shown are typical of three independent experiments conducted with 8-10 mice in each group.

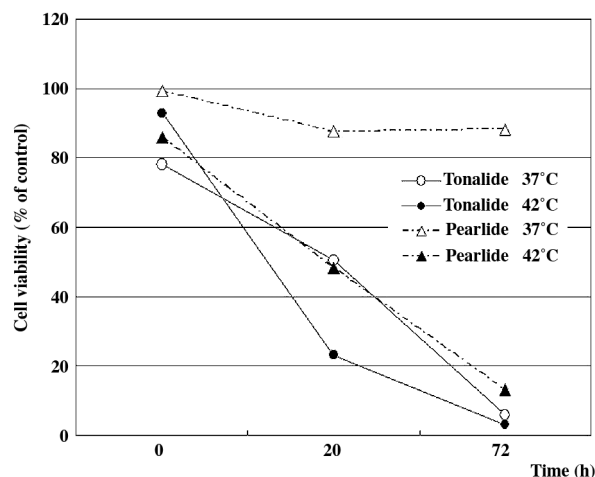


Figure 5. Time course of cytotoxic effects of Tonalide and Pearlide (50  $\mu\text{M}$ ) on Ehrlich ascites tumor (EAT) cells treated at 37 or 42°C and cultured for 20 or 72 h. This illustrates the data presented in Figs. 1-3.

carcinostatic activity in cultured EAT cells implanted in mice, are shown in Fig. 4. Mice that did not receive Tonalide died on average within 15 days, while mice administered a dose of 0.6 or 1.2 mg/day of Tonalide had a lifespan prolonged by up to 17 or 20 days, respectively. With 1.8 mg/day of Tonalide, the lifespan of mice with transplanted tumors was prolonged the longest, for up to 28 days.

## Discussion

Aromatics such as musks have been reported to exhibit anti-tumor activity (1-3). We found delta-alkyllactones and macrocyclic lactones in diverse aromatics as part of a series screening anticancer agents (4,5). The activities of these are enhanced by combination with hyperthermia (5). In the present study, we found that both Tonalide and Pearlide had carcinostatic activities, although those of Tonalide were markedly



stronger than those of Pearlde. These effects were synergistically increased by hyperthermia and influenced by the duration of culture after hyperthermic treatment (Fig. 5). Immediately following treatment, neither of the aromatics had an effect on EAT cells, even after heating at 42°C. Tonalide resulted in an increased carcinostatic effect with the elongation of the culture period, which was enhanced by hyperthermia. The effects of Pearlde were remarkably promoted by the simultaneous combination of hyperthermia and elongation of culture period, but not by the latter alone. This suggests that Pearlde is not decomposed into an inactive form even after long-term culture at 42°C.

Tonalide and Pearlde have similar chemical structures. Imparity in the carcinostatic activity of Tonalide and Pearlde was therefore explained as follows. A position in a molecule of an oxygen atom and a pie electron effect are considered to be important to the structure-activity relationship. The configuration of the O (equal as follows) atom of the carbonyl, which connects chlorobenzene with an indole ring turned to an indole ring CH<sub>3</sub> side, contributes to the anti-inflammatory activity of indomethacin. The sixth place of naphthalene of Tonalide acetylates, the O atom of the acetyl group and CH<sub>3</sub> of an opposite side are in an equal side (configuration displayed in PubChem Compound) and condensation benzene ring and a pie electron of tetrahydronaphthalene configuration conjugate. This configuration performs coaction (a non-covalent bond) in the DNA of a cancer cell, and thereby cellular DNA synthesis is said to be inhibited. In contrast, Pearlde is thought to be inert, whereas the compound becomes closed anulus with an O atom in hexopyranose carbuncle of saccharides. Pearlde exhibits marked carcinostatic activity in combination with hyperthermia, whereas it is almost ineffective at 37°C. It is considered in the benzopyran configuration of pearlde that the pie electron, which exhibits low activity under usual conditions, is activated by heat, often forming ion radicals.

Whether Tonalide and Pearlde have harmful effects remains to be investigated in animal experiments, as they have been reported to be toxic (9-11). Tonalide is not expected to be very harmful at low doses due to its prolonging effect in tumor-transplanted mice. The aromatic exhibited marked carcinostatic activity in cultured tumor cells that was considerably enhanced by hyperthermia, and tumoricidal action in tumor-bearing experimental animals. Thus, it is an attractive and potentially potent antitumor agent in terms of its reducing abilities and side-effects in normal tissue.

## Acknowledgements

We would like to thank Mr. Kunihiro Kinoshita, the managing director, Dr Nobuhiko Ito and Mr. Hiroshi Tsuji (Soda Aromatic Co., Ltd.) for synthesizing and supplying Tonalide and Pearlde.

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