Thermosensitization and induction of apoptosis or cell-cycle arrest via the MAPK cascade by parthenolide, an NF-κB inhibitor, in human prostate cancer androgen-independent cell lines

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Received June 7, 2011; Accepted July 14, 2011

DOI: 10.3892/ijmm.2011.760

Abstract. Parthenolide (PTL), a nuclear factor-κB (NF-κB) inhibitor, has a significant thermo-enhancement effect. Modification of thermosensitivity by treatment with PTL prior to hyperthermia was investigated in the human prostate cancer androgen-independent cell lines PC3 and DU145. In addition, we analyzed the mechanisms related to induction of apoptosis or G_2/M cell-cycle arrest via the effects of ERK1/2, p38 and SAPK/JNK signaling on mitogen-activated protein kinase (MAPK). Lethal damage caused by mild hyperthermia at 41.0˚C or 42.0˚C in both cell lines resulted in a low level of thermosensitivity, while sequential combination with PTL showed significant thermosensitization. Step-up hyperthermia (SUH) (42˚C for 30 min, 43.0˚C or 43.5˚C for various periods) reduced the thermosensitivity of the cells to second heating. However, PTL given as pre-treatment prior to SUH prevented SUH-induced thermal tolerance and resulted in significant thermosensitization. Induction of apoptosis by the combination of PTL and hyperthermia at 44.0˚C was determined by the ratio of sub-G_1 division cells using flow cytometry, which was increased significantly in comparison with single treatment, and was more effective in PC3 than DU145 cells. The behavior of ERK1/2, p38, and SAPK/JNK signaling in the MAPK cascade by treatment with PTL and hyperthermia were examined by Western blotting. As for PC3 cells, ras-downstream p-ERK1/2 was activated and p-p38 slightly activated by combined treatment with PTL and hyperthermia in comparison with each alone. As for DU145 cells, ERK1/2 was not changed, while p38 and SAPK/JNK were slightly activated by combination treatment. These results were related to increases in the induction of apoptosis, G_2/M cell cycle arrest, and lethal damage of cells via the MAPK cascade. Together, our findings demonstrate that PTL is an effective thermosensitizing agent for multidisciplinary therapy for human prostate cancer.

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Key words: parthenolide, hyperthermia, thermosensitization, step-up heating, apoptosis, cell-cycle arrest, NF-κB inhibitor, MAPK cascade, ERK1/2, p38, SAPK/JNK, PC3 cells, DU145 cells

Introduction

When exposed to hyperthermic conditions, most cancer cells acquire thermoresistance or thermotolerance similar to other treatments, such as radiotherapy and chemotherapy. Notably, step-up hyperthermia (SUH), an initial heating of low temperature followed immediately by a second heating of high temperature, induces thermotolerance in contrast to step-down hyperthermia (SDH) which enhances thermosensitivity (1-3). Lindegaard *et al* (4) investigated SDH and SUH preheated at 44.5˚C for 5 min and 41.0˚C for 120 min, respectively. When combined with variable heating at 43.5˚C, the latter pre-treatment for 120 min reduced heat sensitivity to subsequent heating at 43.5˚C, while the former 5 min pre-treatment induced a small degree of thermosensitization when given immediately before the subsequent heating and induced a small degree of thermotolerance when there was a 2 h interval between the treatments. In our previous study, it was revealed that cells treated with 42˚C of hyperthermia alone developed thermotolerance after 2 to 3 h of heating, while SUH (42-44˚C) resulted in thermoresistance to second heating, and SUH combined with adriamycin, a topoisomerase II inhibitor, significantly reduced thermoresistance. These results were correlated with inhibition of heat-induced hsp72 and induced *p53*-dependent apoptosis as reported elsewhere (5). In the present study, SUH (42˚C for 30 min, 43.0 or 43.5˚C for various sequential periods) along with pre-treatment with parthenolide (PTL) prior to SUH were investigated. High hyperthermia has beneficial effects on cells *in vitro*, though such treatment is impractical for clinical cancer therapy, as there are limits in regard to dose and time exposure

before the patient experiences pain. If an appropriate medicinal sensitizer could be applied in combination with thermotherapy, synergistic or at least additive thermo-enhancement effects may be obtained.

It has been reported that PTL inhibits the activation of nuclear factor-κB (NF-κB) transcription (6-8). We previously found that combination therapy with PTL and hyperthermia at 40, 42 or 44˚C caused significant thermosensitization in human lung adenocarcinoma A549 cells with wild-type *p53*, which was due to *p53*- and hsp72-independent apoptosis induction via the NF-κB signaling pathway (9). Another study noted that the cell phase response to PTL in A549 cells synchronized in the G_1/S border phase with hydroxyurea showed remarkable cytotoxicity in the S phase in the same manner as with hyperthermia (10). The ratio of cell-cycle distribution shown by flow cytometric analysis was also increased in sub-G_1 division classified apoptotic cells. Thus, combination therapy of PTL and hyperthermia resulted in significant thermosensitization in A549 cells, which occurs by free apoptosis induction or cell-cycle arrest in the G_2/M phase by inhibiting NF-κB activation.

Just as the heat-shock protein (HSP) family members are known to be thermotolerance inducers, Ras is also known as a gene that causes thermoresistance via various signaling cascades in hyperthermia. It has been reported that the Ras-cdc24 signal transduction pathway mediates thermotolerance (11). In addition, the relationship between thermotolerance and the Ras gene has been reported, as thermotolerance or thermoresistance developed after blocking activation of the Ras-cAMP or mitogen-activated protein kinase (MAPK) pathway (12-14). Ras was first identified as a gene related to virus-induced malignant tumors (15). The Ras gene (or the ras proteins) transmits signals along the MAPK cascade, and plays a part in cell growth or cell mitosis (16). Three distinct groups of MAP kinases have been identified in mammalian cells, extracellular-regulated kinase (ERK), stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), and p38 (17-20), which are mediators of signal transduction from the cell surface to the nucleus (21). c-Jun/AP-1 activation has been implicated in various, often opposing, cellular responses. For example, though there is considerable evidence that c-Jun activation can be a positive step in events leading a cell towards apoptosis, there are also many reports of the opposite; i.e., under certain circumstances c-Jun can inhibit apoptosis and promote proliferation or differentiation instead and these responses are important for normal mammalian development (22). The SAPK/JNK signaling pathway also regulates cellular proliferation, apoptosis, and tissue morphogenesis (23). Xia *et al* (24) reported opposing effects of ERK and SAPK/JNK-p38 MAP kinase on apoptosis, thus the dynamic balance between growth factor-activated ERK and stress-activated SAPK/JNK-p38 pathways may be important in determining whether a cell survives or undergoes apoptosis. Various studies regarding the relationship between Ras and NF-κB have also been reported. Previous experiments showed that ultrasound radiation stimulates NF-κB activation via the Ras/Raf/MEK/ERK signaling pathway (25), while another study found that inhibition of ionizing radiation (IR)-induced activation of NF-κB, but not of either Akt or MAPK kinase, increased the radiosensitization of Ki-Ras transformed human prostate epithelial K-ras cells (26). In addition, oncogenic Ki-Ras induces transactivation of NF-κB through the NF-κB-inducing kinase-IKKβ-IκBα pathway (27). In the present study, we clarified that PTL and hyperthermia have antitumor effects via apoptosis or cell-cycle arrest. The related mechanisms were also examined regarding involvement of the ras/MAPK cascade in the development of thermotolerance via the ERK, SAPK/JNK, and p38 MAPK signal transduction pathways upstream of NF-κB.

Materials and methods

Cells and culture medium. DU145 and PC3 cells from a human androgen-independent prostate cancer cell lines were cultured in RPMI-1640 medium (Invitrogen; Grand Island, NY, USA) and F-12K medium (Invitrogen) under conventional conditions at 37˚C in a humidified incubator with 5% CO_2 in 95% air, respectively (28,29). The RPMI-1640 medium was supplemented with 10% fetal bovine serum (ICN Biomedicas, Inc., OH, USA), 1% MEM NEAA solution (Invitrogen), 1% MEM vitamin solution (Invitrogen), 1% sodium pyruvate and 1% PC and SM mix (Nacalai Tesque, Inc., Kyoto, Japan). The F-12K medium was supplemented with 10% fetal bovine serum and 1% PC and SM mix.

PTL, hyperthermia, and combination treatments. The sesquiterpene lactone PTL (MP Biomedicals, LLC, Solon, OH) was dissolved in culture medium to an appropriate final concentration (2.0 μM) prior to being used for treatment as PTL solution. Cells adhered to the inner side of the bottom of the culture flasks were exposed to PTL by replacement with 6 ml of PTL solution for various time periods. The cells were treated with 2.0 μM of PTL for 6 h, which resulted in 50% lethal damage (LD_{50}). The PTL solution was chased and adhered cells were gently rinsed twice with culture medium containing 3% serum, then re-fed with 6 ml of F-12K or RPMI-1640 medium at 37˚C. Hyperthermia was performed by immersing culture flasks equipped with tightened screw tops in a temperature-regulated water bath (Model EPS-47, Tokyo Seisakusho Co., Tokyo, Japan) preset to the desired temperature, which was maintained within ±0.05˚C, as measured by a thermistor (Model D116-1251, Takara Thermistor Instructions Co., Yokohama, Japan). For the combination treatment, applications of PTL and heating were performed sequentially, during which the cells were exposed to PTL for 6 h, then rinsed twice with culture medium containing 3% serum, placed in F-12K or RPMI-1640 medium, and subjected to hyperthermia. Kinetic assessments of the sensitivity of PC3 and DU145 cells to PTL and hyperthermia were carried out by colony forming assays, and the results were corrected based on the plating efficiency of the control cells (i.e., 80-90%). The average colony multiplicity was <1.1. The T_0 value, which has been adopted as the criterion of cellular thermo- and herbal medicinal-sensitivity, represented the treatment period required to reduce survival by 1/e in the exponentially regressing portion of the survival curve, i.e., the linear portions of the treatment period shown in semilogarithmic survival curves.

Assays of apoptosis and cell-cycle distribution. The kinetics of apoptosis induction, and G_1 and G_2/M cell-cycle arrest of PC3 and DU145 cells following treatment with PTL, hyperthermia, and those in combination were analyzed by flow cytometry. After

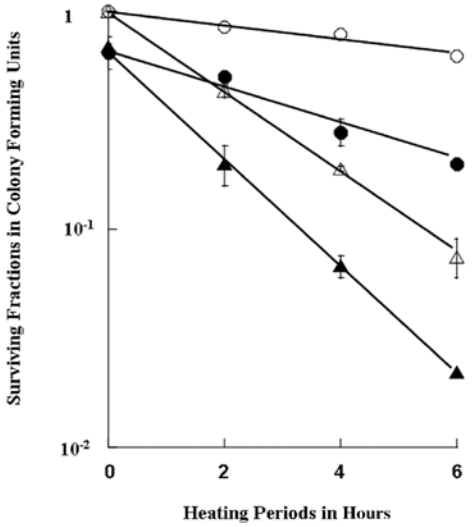

Figure 1. Thermo-enhancement effects of PTL (2.0 μM) on PC3 cells. Ordinate, log surviving fractions (SFs) expressed in colony forming units; Abscissa, 41.0˚C or 42.0˚C heating periods in hours. Open circles, 41.0˚C hyperthermia alone. Open triangles, 42.0˚C hyperthermia alone. Closed circles, PTL for 6 h followed by 41.0˚C hyperthermia for graded periods. Closed triangles, PTL for 6 h followed by 42.0˚C hyperthermia for graded periods. Symbols with vertical bars represent means with standard deviation obtained from at least three data sets. Symbols without a bar represent the standard error within the symbols.

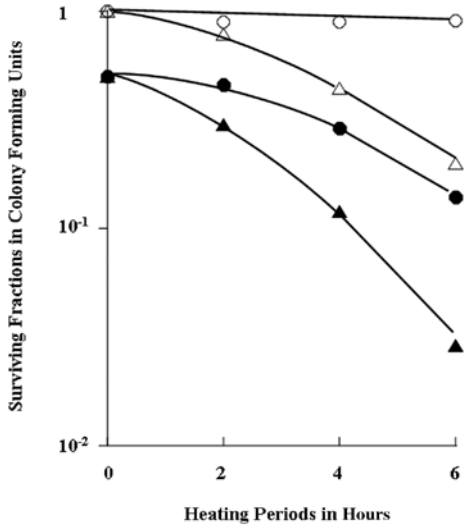

Figure 2. Thermo-enhancement effects of PTL (2.0 μM) on DU145 cells. Ordinate, log surviving fractions (SFs) expressed in colony forming units; Abscissa, 41.0˚C or 42.0˚C heating periods in hours. Open circles, 41.0˚C hyperthermia alone. Open triangles, 42.0˚C hyperthermia alone. Closed circles, PTL for 6 h followed by 41.0˚C hyperthermia for graded periods. Closed triangles, PTL for 6 h followed by 42.0˚C hyperthermia for graded periods. Symbols with vertical bars represent means with standard deviation obtained from at least three data sets. Symbols without a bar represent the standard error within the symbols.

0, 24, 48 and 96 h of incubation at 37˚C after various treatments, cells ($1x10^5$) were harvested by trypsinization, re-suspended in culture medium, rinsed twice with ice-cold PBS(-), and fixed in ice-cold ethanol at a rate of 7 after PBS(-) was added at a rate of 3 into culture tubes. The cells were stored at 4˚C for at least 24 h, then collected by centrifugation, rinsed twice with ice-cold PBS(-), and treated with 1 mg/ml of RNase A (Type II-A, Sigma-Aldrich Corp.) at room temperature for 30 min. Cells were then stained with 100 μg/ml of propidium iodide (PI) (Sigma-Aldrich Corp.) for at least 30 min on ice in the dark. The cell-cycle distribution of cells was analyzed using a flow cytometer (Beckman Coulter, Inc., Fullerton, CA). Immediately before analysis, cell suspensions were filtered though 40-μm diameter nylon meshes to remove cell aggregate and debris. Ten thousand events per determination were analyzed for each sample and quantification of the cell-cycle distribution was performed using software provided by the manufacturer. A representative histogram of the population distribution in regard to the cell-cycle status based on DNA content is presented.

Examination of MAPK cascades by Western blotting. Intracellular protein and phosphorylation of ERK1/2, SAPK/JNK and p38 MAPK after application of PTL, heating, and those combined in PC3 or DU145 cells ($1x10^6$) were examined by Western blotting. Cells were harvested by trypsinization and re-suspended in F-12K or RPMI-1640 medium. After rinsing with ice-cold PBS(-) twice, they were dissolved in RIPA lysis buffer, then treated by freezing at -20˚C and thawing on ice for 3 times. Cell lysates were centrifuged at 14,000 rpm at 4˚C for 10 min to remove cell debris. Next, the supernatants were diluted by half with SDS-PAGE, and subjected to a block incubator at 95˚C for 3 min after stirring with a vortex and transformed. The protein contents of the supernatants were quantified using a protein assay kit (Bio-Rad Laboratories, Richmond, CA). Aliquots of protein (10 μg) were subjected to Western blotting for ERK1/2, SAPK/JNK, p38 and their phosphorylated antibodies (Cell Signaling Technology, Japan KK). After electrophoresis on 10% polyacrylamide gels containing 0.1% solution dodecyl sulfate (SDS) and electrophoretic transfer onto Immobilon-P, PVDF-membranes (Millipore Corp., Bedford, MA), the membranes were incubated with phosphorylated-ERK1/2, SAPK/JNK and p38 MAPK. β-actin (Cell Signaling Technology) served as a loading control (30).

Results

Thermosensitization effects of PTL with hyperthermia at 41 and 42˚C. The thermo-enhancement effects of PTL were investigated based on survival curves produced after combination therapy with 2.0 μM of PTL for 6 h and hyperthermia at 41 or 42˚C using PC3 and DU145 cells, as shown in Figs. 1 and 2. The survival curves at 41˚C alone up to 6 h for both cell lines were straight slopes, which indicated slight heat-induced cell death. Cellular lethal thermosensitivity was estimated based on the T_0 value (heating time required to reduce survival by 1/e), which was reciprocal to the straight slope of the survival curve in the exponential phase (31,32), as shown in Table I. When PC3 and DU145 cells were heated at 41˚C for various periods up to 6 h, both cell lines showed slight thermosensitivity with T_0 values of 9.5 and 25.2 h, respectively. With 42˚C hyperthermia alone, DU145 cells showed slight thermotolerance after 2-4 h of heating, while PC3 cells showed a higher level of thermosensitivity. When PC3 cells were treated with PTL (2.0 μM) for 6 h prior to hyperthermia at 41 or 42˚C, the survival curves were straight, as PTL showed synergistic thermo-enhancement effects of 5.4 h (T_0 value) to 41˚C and

Table I. T_0 values of two cell lines for survival from treatments.

Cell line		Hyperthermia alone, T_{01}	PTL (6 h) and hyperthermia, T_{02}	Enhancement ratio, T_{01}/T_{02}
PC3	41˚C	9.5 h	5.4 h	1.76
	42˚C	4.2 h	3.1 h	1.36
DU145	41˚C	25.2 h	6.3 h	4.00
	42˚C	4.6 h	3.2 h	1.44

The thermo-enhancement effects of thermotherapy with 2.0 μM PTL were compared based on the T_0 values. PC3 and DU145 cells were treated with PTL (2.0 μM) for 6 h followed by hyperthermia at 41 or 42˚C in the combined treatment.

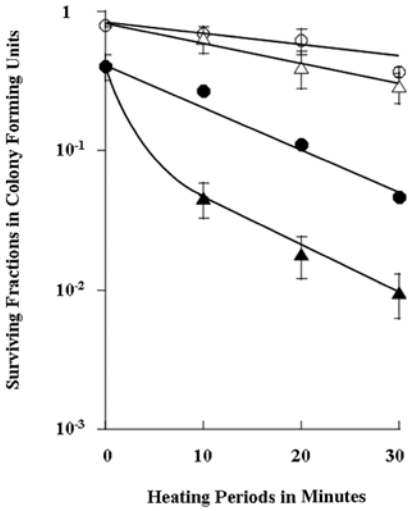

Figure 3. Thermo-enhancement effects of PTL (2.0 μM) treated prior to step-up hyperthermia (SUH) on PC3 cells. Ordinate, log surviving fractions (SFs) expressed in colony forming units; abscissa, heating periods at 43.0˚C or 43.5˚C in minutes. Open circles, SUH (42.0˚C, 30 min - 43.0˚C for graded periods). Open triangles, SUH (42.0˚C, 30 min - 43.5˚C for graded periods). Closed circles, PTL for 6 h prior to SUH (42.0˚C, 30 min - 43.0˚C for graded periods). Closed triangles, PTL for 6 h prior to SUH (42.0˚C, 30 min - 43.5˚C for graded periods). The open circle and the open triangle on the ordinate represent hyperthermia at 42.0˚C alone for 30 min. The closed circle and the closed triangle on the ordinate represent PTL for 6 h followed by hyperthermia at 42.0˚C for 30 min. Symbols with vertical bars represent means with standard deviation obtained from three independent SFs. Symbols without a bar represent the standard error within the symbols.

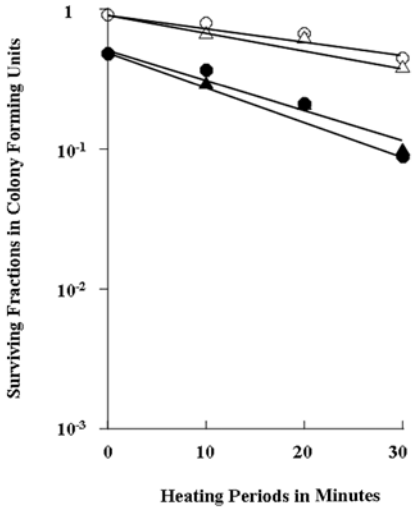

Figure 4. Thermo-enhancement effects of PTL (2.0 μM) treated prior to SUH on DU145 cells. Ordinate, log surviving fractions (SFs) expresed in colony forming units; abscissa, heating periods at 43.0˚C or 43.5˚C in minutes. Open circles, SUH (42.0˚C, 30 min - 43.0˚C for graded periods). Open triangles, SUH (42.0˚C, 30 min - 43.5˚C for graded periods). Closed circles, PTL for 6 h prior to SUH (42.0˚C, 30 min - 43.0˚C for graded periods). Closed triangles, PTL for 6 h prior to SUH (42.0˚C, 30 min - 43.5˚C for graded periods). The open circle and the open triangle on the ordinate represent hyperthermia at 42.0˚C alone for 30 min. The closed circle and a closed triangle on the ordinate represent PTL for 6 h followed by hyperthermia at 42.0˚C for 30 min. Symbols with vertical bars represent means with standard deviation obtained from three independent SFs. Symbols without a bar represent the standard error within the symbols.

3.1 h (T_0 value) to 42˚C on PC3 cells. Similarly, when DU145 cells were treated in combination with PTL and hyperthermia at 41 and 42˚C, slight thermotolerance appeared after 2-4 h of heating, even when pre-treated with PTL. However, with longer periods of heating, the cells showed synergistic thermo-enhancement effects of 6.3 h (T_0 value) to 41˚C and 3.2 h (T_0 value) to 42˚C.

Thermosensitization effects of PTL with SUH. A previous study found that thermal resistance was induced with SUH, while the thermal enhancement effects of SUH were lower in comparison with those caused by SDH (33,34). In our study, the thermo-enhancement effects and suppression of thermal resistance by PTL on SUH (42˚C for 30 min - 43.0 or 43.5˚C for various periods, in sequence) were investigated by comparing dose-response curves for SUH alone and sequential treatments with 2.0 μM of PTL for 6 h prior to SUH in PC3 and DU145 cells (Figs. 3 and 4). Thermosensitivity of PC3 cells treated by SUH alone was moderate, and thermotolerance was not induced by post-heating at 43.0 or 43.5˚C. Furthermore, combination treatments with PTL and SUH performed sequentially showed significant thermo-enhancement effects, especially with SUH at 43.5˚C post-heating, which showed markedly synergistic enhancement effects over the 10 min heating periods (Fig. 3). Similarly, the thermo-enhancement effects obtained by the combination of PTL and SUH in DU145 cells were also investigated. The susceptibility to thermosensitivity of DU145 cells with SUH alone was approximately the same as that of PC3 cells with post-heating of 43.0 or 43.5˚C (Fig. 4). In addition, the thermo-enhancement effects by the combination of PTL and SUH with 43.0˚C post-heating on DU145 cells were

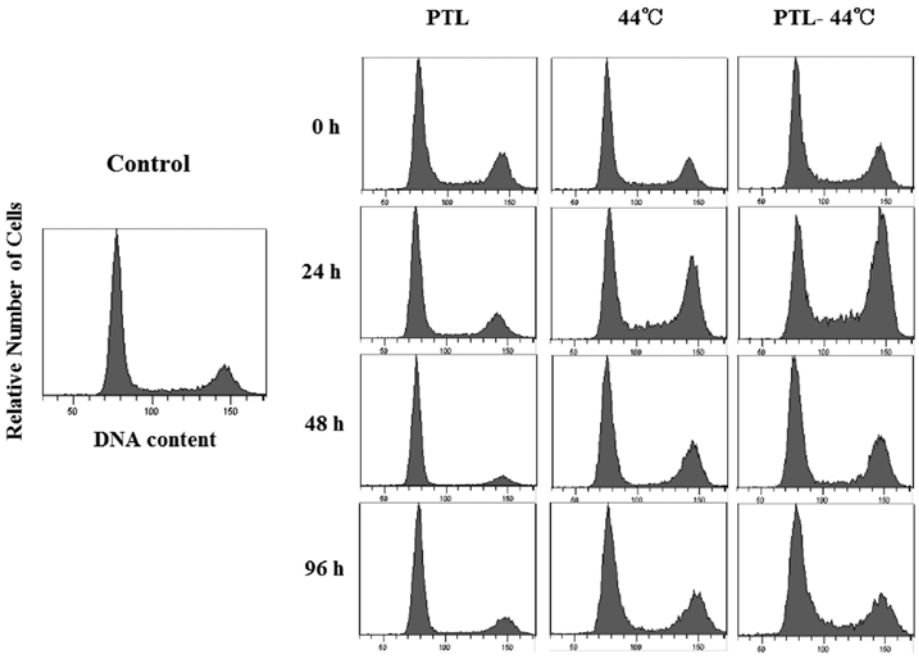

Figure 5. Flow cytometric determination of cell cycle distribution at 0, 24, 48 and 96 h of incubation at 37˚C after treatment of PC3 cells with PTL (2.0 μM) at 37˚C for 6 h, hyperthermia at 44˚C for 30 min, or a sequential combination of both. Ordinates, relative number of cells; Abscissae, DNA contents through fluorescence intensity with propidium iodide staining. The panels show representative profiles of treated and untreated (control) cells for two or three independent experiments.

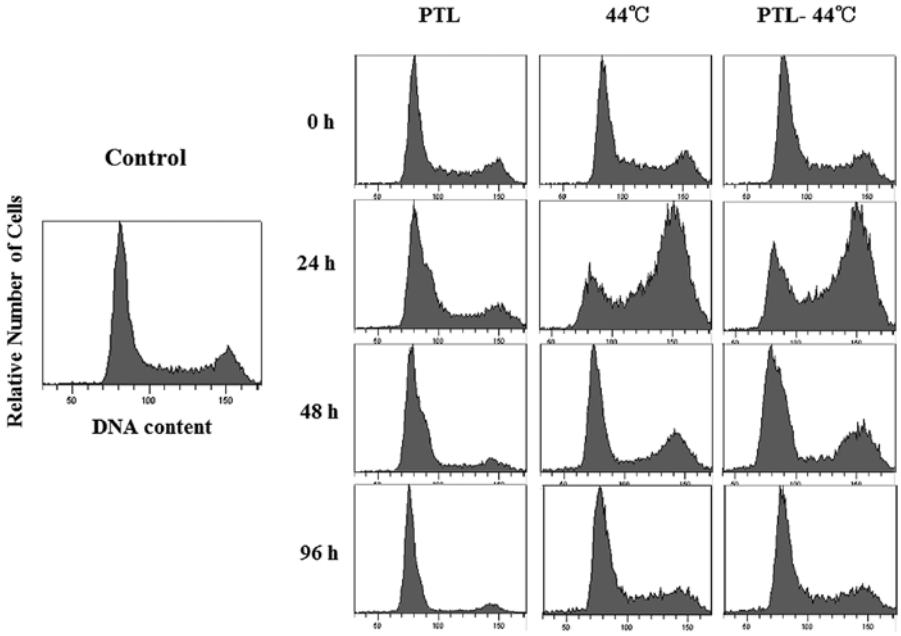

Figure 6. Flow cytometric determination of cell cycle distribution at 0, 24, 48 and 96 h of incubation at 37˚C after treatment of DU145 cells with PTL (2.0 μM) at 37˚C for 6 h, hyperthermia at 44˚C for 30 min, or a sequential combination of both. Ordinates, relative number of cells; Abscissae, DNA contents through fluorescence intensity with propidium iodide staining. The panels show representative profiles of treated and untreated (control) cells for two or three independent experiments.

significant, while the effects of 43.5˚C post-heating on DU145 cells were moderate as compared to those on PC3 cells (Fig. 4).

Apoptosis and cell-cycle distribution assays. We examined the induction of apoptosis and cell-cycle arrest based on the cell-cycle distribution by flow cytometry to elucidate the possible mechanism of thermosensitization by sequential combination of PTL and hyperthermia at 44˚C in PC3 and DU145 cells. The cell-cycle distribution in both cell lines was evaluated at various periods after treatment with 2.0 μM PTL for 6 h, heating at 44˚C for 30 min, and those in combination. Representative histograms showing the population distribution in regard to the

Table II. Distribution (%) of cell population in the phases of the cell cycle after the treatment of PC3 and DU145 cells.

Cell-cycle distribution	Interval periods (h)	Control	PTL	44˚C	PTL/44˚C
PC3 cells					
sub-G_1	0		1.96±1.10	1.75±0.32	4.00±0.20
	24		2.85±1.35	2.60±1.10	5.81±1.05
	48	0.60±0.02	1.30±0.00	3.20±0.65	4.30±1.30
	96		5.70±1.90	3.20±0.00	6.90±1.30
	average		2.95±1.09	2.70±0.78	5.25±0.96
G_1	0		53.3±5.80	58.0±6.53	52.6±4.73
	24		55.7±0.15	43.5±5.97	36.1±6.43
	48	45.8±1.45	56.6±1.47	60.6±3.60	53.8±0.70
	96		70.0±2.55	53.9±1.70	46.7±2.65
	average		58.9±2.49	54.0±4.45	47.3±3.62
G_2/M	0		24.2±0.15	22.3±2.75	29.7±1.60
	24		19.0±0.67	34.1±5.22	40.7±5.34
	48	19.8±1.70	14.0±2.25	24.3±4.50	27.8±2.60
	96		15.1±2.90	22.7±3.70	23.0±0.25
	average		18.1±1.49	25.9±4.04	30.3±2.45
DU145 cells					
sub-G_1	0		2.50±0.67	1.87±0.57	2.41±0.58
	24		2.71±0.13	2.35±0.43	4.53±0.10
	48	1.10±0.02	3.50±0.80	5.50±0.00	6.70±1.10
	96		3.20±1.85	7.60±0.60	10.60±0.80
	average		3.44±0.86	4.33±1.13	6.06±0.65
G_1	0		55.5±0.85	58.1±9.55	59.1 ±7.83
	24		53.4±5.86	44.0±4.35	39.1±1.55
	48	51.4±5.62	64.7±6.95	53.8±10.25	50.4±8.95
	96		75.7±6.00	57.2±9.6	53.9±8.50
	average		62.3±4.91	53.3±8.43	50.7±6.70
G_2/M	0		19.4±2.05	21.4±1.05	20.1±0.70
	24		17.8±1.60	46.4±5.94	36.0±7.02
	48	16.3±2.31	9.00±0.75	19.8±2.05	24.7±0.10
	96		7.90±1.80	13.4±1.40	15.0±2.15
	average		13.5±1.55	25.3±2.34	24.0±2.49

cell-cycle status based on DNA content as measured by PI are shown in Figs. 5 and 6. Distribution of cells in the sub-G_1 phase (apoptotic cell population), and 2 peaks of G_1 and G_2/M are represented as a percentage (%) of the coefficient of variation (CV) values presented in Table II. First, apoptosis induction in PC3 cells is represented by the average up to 96 h in the sub-G_1 phase, which was 0.60±0.02% for the control, 2.95±1.09% for PTL alone, 2.70±0.78% for 44˚C alone, and 5.25±0.96% for the combination of PTL and 44˚C (Table II). Similarly, apoptosis induction in the DU145 cells was 1.10±0.02% for the control, 3.44±0.86% for PTL alone, 4.33±1.13% for 44˚C alone and 6.06±0.65% for the combination (Table II). With the combination, PTL significantly enhanced heat-induced apoptosis in both PC3 and DU145 cells, which was about 1.9- and 1.4-fold greater than heating alone in PC3 and DU145 cells, respectively. Figs. 7 and 8 show kinetic apoptosis induction up to 96 h after treatment, based on the value (%) corresponding to the sub-G_1 phase. The G_2/M phase distribution for PC3 cells showed a significant increase following treatment with PTL alone (18.1±1.49%), 44˚C alone (25.9±4.04%), and those in combination (30.3±2.45%) in comparison with the control (19.8±1.70%) (Table II). The G_2/M phase distribution for DU145 cells also showed a significant increase following treatment with PTL alone (13.5±1.55%), 44˚C alone (25.3±2.34%), and those in combination (24.0±2.49%) in comparison with the control (16.3±2.31%) (Table II). The distribution of cells in the G_2/M phase in both cell lines was remarkably increased at 24 h after treatment with 44˚C heating and that combined with PTL (Figs. 5 and 6). These results indicate that treatment with PTL, heating at 44˚C, and particularly those in combination contribute to the effects of thermo-enhancement on cell-cycle arrest in G_2/M as well as apoptosis induction.

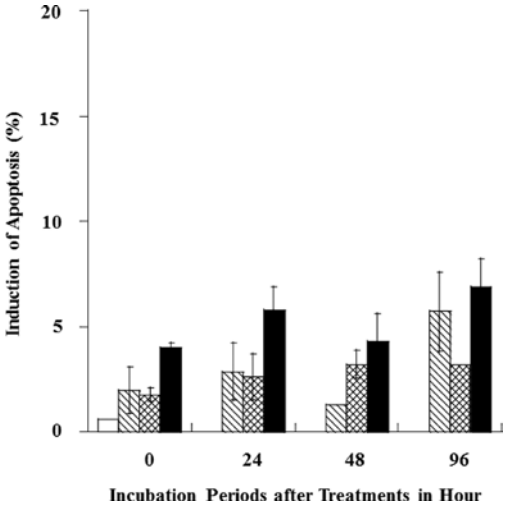

Figure 7. Estimated quantifications of the sub-G_1 phase in cell-cycle distribution represent induced apoptosis, obtained by flow cytometry on PC3 cells. Ordinate, relative number of apoptotic cells over the totals are shown by percentage: abscissa, 37˚C incubation periods in hours after treatment. Columns represent the following groups: blank, control; slanting stripes, PTL (2.0 μM) for 6 h; checkered, 44˚C for 30 min; and black, combination of both.

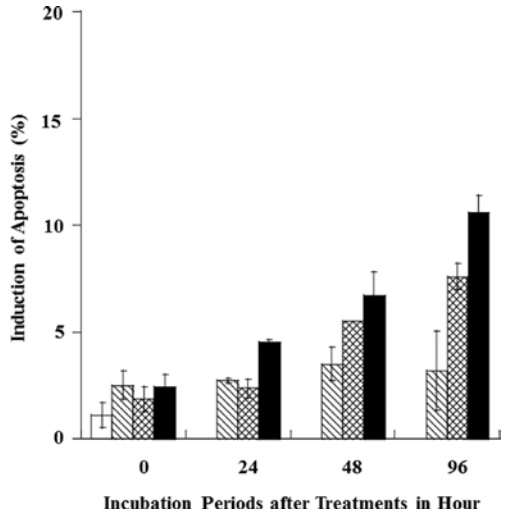

Figure 8. Estimated quantifications of the sub-G_1 phase in cell-cycle distribution represent induced apoptosis, obtained by flow cytometry on DU145 cells. Ordinate, relative number of apoptotic cells over the totals are shown by percentage: abscissa, 37˚C incubation periods in hours after treatment. Columns represent the following groups: blank, control; slanting stripes, PTL (2.0 μM) for 6 h; checkered, 44˚C for 30 min; and black, combination of both.

Examination of the MAPK cascade with PTL and heating by Western blotting. Activated protein and the phosphorylation of ERK1/2, p38 and SAPK/JNK on the MAPK cascade were assayed according to time course (0, 24, 48 and 96 h) after treatment with 2.0 μM of PTL for 6 h, heating at 44˚C for 30 min, and those in combination by Western blotting. These results were used to investigate whether apoptosis induction or cell-cycle arrest is linked via the 3 cascades to Ras/Raf/MAPK signaling upstream of NF-κB in PC3 and DU145 cells (Figs. 9 and 10). In PC3 cells, the ERK1/2 dimer was inactivated during the latter half after treatment with PTL alone, while this inactivation occurred at the initial point after treatment with 44˚C alone or those in combination (Fig. 9). p-ERK1/2

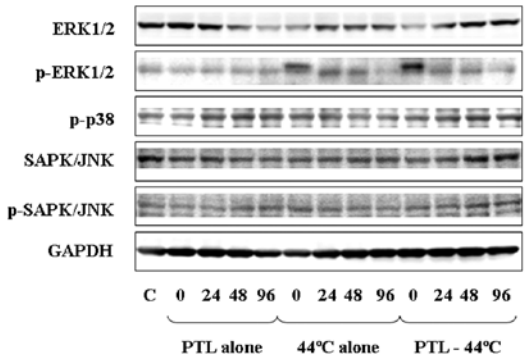

Figure 9. Western blot analysis of cellular amounts of ERK1/2, p-ERK1/2, p-p38, SAPK/JNK, p-SAPK/JNK at various periods after treatments with PTL (2.0 μM) for 6 h, 44˚C hyperthermia for 30 min and the combination in PC3 cells. Numbers 0, 24, 48 and 96 on the horizontal line represent 37˚C incubation intervals in hours after treatments and the blotting, respectively. C, on the left represents the lane of the untreated control.

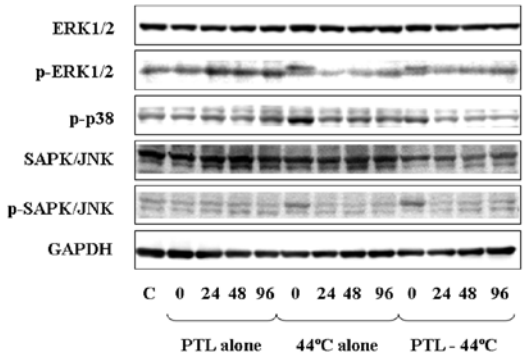

Figure 10. Western blot analysis of cellular amounts of ERK1/2, p-ERK1/2, p-p38, SAPK/JNK, p-SAPK/JNK at various periods after treatments with PTL (2.0 μM) for 6 h, 44˚C hyperthermia for 30 min and the combination in DU145 cells. Numbers 0, 24, 48 and 96 on the horizontal line represent 37˚C incubation intervals in hours after treatments and the blotting, respectively. C, on the left represents the lane of the untreated control.

in PC3 cells was activated by 44˚C alone and remarkably activated after treatment with the combination, while p-p38 in PC3 cells was slightly activated by both PTL alone and the combination (Fig. 9). Also, SAPK/JNK was slightly activated by both the single and combination treatments, though that expression was increased during the latter half after treatment with the combination. The amount of p-SAPK/JNK in PC3 cells was slight with the combination in comparison to that with the control (Fig. 9). In PC3 cells, the NF-κB inhibitor PTL combined with hyperthermia slightly activated 3 MAPK family signaling pathways (ERK1/2, SAPK/JNK and p38), of which p-ERK1/2 and p-p38 were activated to the greatest degree. Thus, we considered that apoptosis induction or cell-cycle arrest in G_2/M was mostly obtained via ERK1/2 and p38. As for DU145 cells, ERK1/2 was not changed in comparison with the control with any of the treatments or at any time point after treatment (Fig. 10). p-ERK1/2 in DU145 cells was slightly activated by PTL alone, but not by 44˚C alone or those in combination. p-p38 in DU145 cells was also slightly activated by PTL or 44˚C alone, and was scarcely detected only immediately after treatment by the combination. SAPK/JNK in

DU145 cells was induced by PTL or 44˚C alone, but suppressed by the combination. Finally, p-SAPK/JNK in DU145 cells was activated only at 0 h after 44˚C alone and the combination. Thus, the NF-κB inhibitor PTL in combination with hyperthermia activated p-SAPK/JNK in DU145 cells mainly via MAPK family signaling pathways (ERK1/2, SAPK/JNK, p38), indicating that apoptosis induction or cell-cycle arrest in G_2/M was mostly via SAPK/JNK.

Discussion

When thermal sensitizers are used in clinical anticancer therapy to synergistically produce thermosensitivity or significantly reduce thermotolerance without side effects, they can contribute to multidisciplinary anticancer therapy for affected patients. Previously, we investigated the abilities of various chemicals, including PTL, adriamycin (doxorubicin), bleomycin, cisplatin, amrubicin and its metabolite amrubicinol, in regard to their ability to modify the effects of hyperthermia at kinetic and molecular levels (5,9,10,35,36). Markovic and Stunhlmeier (37) reported that short term hyperthermia prevented the activation of proinflammatory genes in fibroblast-like synoviocytes by blocking the activation of NF-κB. We also previously found that the NF-κB inhibitor PTL in combination with 42˚C heating showed significant thermo-enhancement effects in human lung adenocarcinoma A549 cells, by which apoptosis or cell-cycle arrest was induced though activation of inhibited p-NF-κB (10). In the present study, we investigated the modification of thermosensitivity by PTL and related mechanisms using the human androgen-independent prostate cancer cell lines PC3 and DU145.

Thermo-enhancement effects of PTL with heating at 41 and 42˚C. The survival curves at 41˚C alone up to 6 h in both cell lines revealed slight heat-induced cell death (Figs. 1 and 2). When PC3 and DU145 cells were heated at 41˚C for various periods up to 6 h, the cell lines showed slight thermosensitivity of 9.5 and 25.2 h (T_0 values), respectively (Table I). With the 42˚C condition alone, DU145 cells showed slight thermotolerance during 2-4 h of heating, while PC3 cells showed a higher level of thermosensitivity. When PC3 cells were treated with PTL (2.0 μM) for 6 h prior to hyperthermia at 41 or 42˚C, the survival curves of the cells were straight. Thus, PTL provided synergistic thermo-enhancement effects in PC3 cells. Similarly, when DU145 cells were sequentially treated with the combination of PTL and hyperthermia at 41 or 42˚C, slight thermotolerance appeared during 2-4 h of heating even when pretreated with PTL. However with continued heating, the cells showed significant thermo-enhancement effects.

SUH combined with PTL. With SDH, the first heating at high temperature increased the thermosensitivity of cells to the second heating at low temperature, while with SUH, the primary heating induces thermo-tolerance and reduces thermosensitivity to the second heating (4,33). When SUH is immediately applied after radiation, it has been reported to have slight advantages over SDH, such as tumor growth delay (34). In the present study, the thermo-enhancement effects or suppression of thermal resistance obtained by SUH (42˚C for 30 min - 43.0 or 43.5˚C for various periods, sequence) in combination with PTL were investigated by comparing the dose-response curves for SUH alone with those for sequential treatments with 2.0 μM of PTL for 6 h prior to SUH using the PC3 and DU145 cell lines (Figs. 3 and 4). In PC3 cells, sequential treatment with PTL and SUH showed significant thermo-enhancement effects, especially with SUH (43.5˚C post-heating), which resulted in markedly synergistic enhancement effects after about 10 min of heating (Fig. 3). Similarly, the thermo-enhancement effects of combination therapy with PTL and SUH (43.0˚C post-heating) showed significant effects in DU145 cells as well as PC3 cells, while that with 43.5˚C post-heating had only moderate effects in comparison with PC3 cells (Fig. 4). Combination with PTL prevented SUH-induced thermo-tolerance and resulted in significant thermosensitization, which was more effective in PC3 cells (*p53*-null) in comparison with DU145 cells (*p53*-mutant).

Assays of apoptosis and cell cycle distribution. A number of studies of gene and signaling pathways in relation to induction of apoptosis have been reported. The mechanism of hyperthermia-induced apoptosis was found to be mediated by caspase-3 (38). It was also reported that the PI3K/Akt pathway regulates apoptosis, in which the kinases are either pro-apoptotic or anti-apoptotic, depending upon the types of stimuli and circumstances (39,40). There are other reports showing that inhibition of NF-κB induces apoptosis and cell-cycle arrest in G_2/M through various signaling pathways. In addition, inhibition of radiation-induced activation of NF-κB in prostate cancer cells promoted apoptosis and G_2/M cell-cycle arrest, which were correlated with increased p21/WAF1/Cip1 and decreased cyclin B1 expression (41). The viability of HeLa cells was suppressed by inducing cell-cycle arrest in the G_2/M phase and mitochondrial apoptosis through *p53*-dependent expression of the Bcl-2 family (42). The pathway for NF-κB/caspase activation is independent of the NF-κB/cell-cycle pathway and the events downstream of the NF-κB/caspase-9 cascade lead to apoptosis (43). In the present study, cell-cycle distributions were evaluated at various time points after treatment with the NF-κB inhibitor PTL (2.0 μM) for 6 h, heating at 44˚C for 30 min, and those in combination using PC3 and DU145 cells (Figs. 5 and 6). The distributions of cells in the sub-G_1 (apoptotic cell population), G_1 and G_2/M phases are shown in Table II. For PC3 cells, apoptosis induction is represented by the average value up to 96 h in the sub-G_1 phase, which resulted in 0.60±0.02% for the control, 2.95±1.09% for the PTL-treated cells, 2.70±0.78% for the 44˚C heat-treated cells, and 5.25±0.96% for the combination (Table II). As for the DU145 cells, apoptosis induction is represented by the average up to 96 h in the sub-G_1 division, which resulted in 1.10±0.02% for the control, 3.44±0.86% for the PTL-treated cells, 4.33±1.13% for the 44˚C heat-treated cells, and 6.06±0.65% for the combination (Table II). With the combination therapy, PTL enhanced heat-induced apoptosis significantly in both PC3 and DU145 cells, and the enhancement ratio was about 1.9-fold greater than heating alone in PC3 cells and about 1.4-fold greater than heating alone in DU145 cells. Our findings that the ratio of apoptosis induction in PC3 cells was higher than that in DU145 cells corresponded to the finding that the amount of lethal damage, based on survival curves was more effective in PC3 cells than in DU145 cells. These results may be related to the fact that PC3 cells are null for *p53* (44,45)

and DU145 cells possess mutant *p53* (46,47). Next, the distributions of cells in G_1 and G_2/M arrest among the cell populations after the various treatments are shown numerically in Table II. In PC3 cells, the G_1 arrest phase was most increased by treatment with PTL, while G_2/M arrest showed a significant increase by the combination treatment. In DU145 cells, G_1 arrest showed the greatest increase by PTL, and G_2/M arrest was increased by both 44˚C heating and the combination. These results indicate that PTL and 44˚C heating, and notably those in combination contribute to thermosensitization to induce cell-cycle arrest in the G_2/M phase as well.

Examination of MAPK cascades by Western blotting. The Ras/Raf/MAPK cascade promotes mitogenesis or cell growth via the EGFR signaling pathway, which mainly participates in proliferation, anti-apoptosis, infiltration, and metastasis (48,49). When activation of the Ras/Raf/MAPK cascade is elicited, it in turn initiates NF-κB activation, though that process is inhibited via suppression of ras farnesylation (50-52). p-ERK1 and p-ERK2 constitute the dimers of ERK1/2, which translocate from the cytoplasm to the nucleus and activate the transcription factor (53,54). However, a resent report suggested that ERK causes neuronal apoptosis (55). In the present study, p-ERK1/2 in PC3 cells was activated by 44˚C heating alone and remarkably activated immediately after treatment with heating in combination with PTL (Fig. 9). p-ERK1/2 in DU145 cells was significantly activated by PTL alone, but it was not activated by 44˚C alone or the combination (Fig. 10). p-p38 in PC3 cells was slightly activated by PTL alone and the combination, while p-p38 in DU145 cells was remarkably activated immediately after treatment with 44˚C alone. p-SAPK/JNK in PC3 cells was significantly activated by the combination and p- SAPK/JNK in DU145 cells was significantly activated immediately after treatment with the combination. In addition, MAPK family (ERK, JNK, p38) cascades were activated by treatment with PTL, and 44˚C heating in both cell lines. In PC3 cells, PTL in combination with hyperthermia activated all MAPK phosphorylated cascades increasingly over time, with the most remarkable increases seen in p-ERK1/2 and p-p38, which suggests that thermosensitization by PTL is associated with apoptosis induction or G_2/M cell-cycle arrest mainly via the ERK1/2 and p38 cascades. In DU145 cells, PTL in combination with hyperthermia activated p-p38 and p-SAPK/JNK, which suggests that thermosensitization by PTL is associated with apoptosis induction or G_2/M cell-cycle arrest mainly via the SAPK/JNK cascades. The contrasting results between the cell lines may be due to the fact that PC3 cells are null for *p53* and DU145 cells possess mutant *p53*. With PC3 cells, the thermo-enhancement effects occurred earlier and were more effective than with DU145 cells, which suggests that PTL- and heat-induced apoptosis or G_2/M cell-cycle arrest in PC3 cells without *p53* occurred via ERK1/2 and p38 signaling in the MAPK cascades with up-regulation of NF-κB irrespective of *p53*, while that in DU145 cells possessing mutant *p53* may have been caused by slight *p53*-dependent thermoresistance.

The PC3 and DU145 cell lines have a human prostate epithelial origin, and are composed of androgen-independent cells (56). For prostate cancer therapy, various treatments such as surgery, radiation therapy, stereotactic radiosurgery, proton therapy, chemotherapy, cryosurgery and hormonal therapy have been applied. In the anti-androgen withdrawal syndrome, anti-androgens cause cell growth against the original effects of the medicine (57,58). Our results demonstrate that PTL is an effective thermosensitizer for multidisciplinary anticancer therapy in humans and for the clinical treatment of prostate cancer.

Acknowledgements

The present study was supported in part by a Grant-in-Aid for Scientific Research (C) (no. 22591381) from the Ministry of Education, Science and Culture, Japan, for analysis of the molecular mechanisms related to radiosensitization by the transcription factor NF-κB inhibitor PTL, 2010-2012. Support was also provided in part by a grant of incorporation from Suzuki Urology Promotion Research. We express our gratefulness to Ms. Junko Yamamoto for assistance with measurements and processing of the flow cytometric assay data and with the Western blotting procedures.

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