Sinodielide A exerts thermosensitizing effects and induces apoptosis and G_2/M cell cycle arrest in DU145 human prostate cancer cells via the Ras/Raf/MAPK and PI3K/Akt signaling pathways

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Abstract. Sinodielide A (SA) is a naturally occurring guaianolide, which is isolated from the root of *Sinodielsia yunnanensis*. This root, commonly found in Yunnan province, is used in traditional Chinese medicine as an antipyretic, analgesic and diaphoretic agent. A number of studies have reported that agents isolated from a species of Umbelliferae (Apiaceae) have antitumor activities. We previously reported, using combined treatments with this medicinal herb and hyperthermia at various temperatures, an enhanced cytotoxicity in the human prostate cancer androgen-independent cell lines, PC3 and DU145, and analyzed the related mechanisms. In the present study, we investigated the effects of treatment with SA prior to hyperthermia on the thermosensitivity of DU145 cells, and the mechanisms related to the induction of apoptosis and G_2/M cell cycle arrest via the activation of extracellular-regulated kinase (ERK)1/2, c-Jun N-terminal kinase (JNK) mitogen-activated protein kinase (MAPK) signaling pathways, as well as the phosphoinositide 3-kinase (PI3K)/Akt signaling pathways. Cells were exposed to hyperthermia alone (40-44˚C) or hyperthermia in combination with SA. Lethal damage to cells treated with mild hyperthermia (40 or 42˚C) for up to 6 h was slight; however, hyperthermia in combination with SA synergistically enhanced thermosensivity. Lethal damage to cells treated with acute hyperthermia (43 or 44˚C) was more severe, but these effects were also enhanced and were more significant by the combined treatment with SA. The kinetics of apoptosis induction and cell cycle distribution were analyzed by flow cytometry. In addition, the levels of ERK1/2, JNK and Akt were determined by western blot analysis. The incidence of apoptotic cells after treatment with SA (20.0 μM) at 37˚C for 4 h, hyperthermia (44˚C) alone for 30 min, and the combination in sequence were examined. The sub-G1 division (%) in the diagram obtained by flow cytometry was applied to that assay. The percentage of apoptotic cells (10.53±5.02%) was higher at 48 h as compared to 0, 12 and 24 h after treatment. The distribution of DU145 cells in the G_2/M cell cycle phase was markedly increased after 24 h of heating at 44˚C and after the combined treatment with heating and SA. The phosphorylation of ERK1/2 was reduced following treatment with heating and SA, while the levels of phosphorylated JNK (p-JNK) were markedly increased immediately after heating at 44˚C and when heating was combined with SA. By contrast, the levels of phosphorylated Akt (p-Akt) were immediately increased only after heating at 44˚C. Thus, we concluded that SA exerts its thermosensitizing effects on DU145 cells by inhibiting the activation of the MAPK/ERK1/2 and PI3K/Akt signaling pathways.

Introduction

Sinodielide A (SA) is a naturally occurring guaianolide, which is isolated from the root of *Sinodielsia yunnanensis* (1). This root is used in traditional Chinese medicine as an antipyretic, analgesic and diaphoretic agent. There are a number of studies on the anticancer effects of agents isolated from a species of Umbelliferae (Apiaceae). The major component of *Angelica japonica* roots, 3'-*O*-acetylhamaudol, has dual functions: it has anti-angiogenic functions and can activate intestinal intraepithelial lymphocytes (2). Furthermore, xanthoangelol D, isolated from the roots of *Angelica keiskei*, inhibits endothelin-1

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production by suppressing nuclear factor-κB (NF-κB) (3). When cancer cells are subjected to hyperthermia, they typically acquire thermotolerance during or after the heating process, similar to other treatments, such as radiotherapy and chemotherapy; this is one of the problems associated with thermotherapy. In an attempt to overcome the problems of thermotolerance and drug resistance associated with thermo- and chemotherapy, combined treatment with hyperthermia and chemotherapeutic agents has become widely adopted as a cancer treatment strategy to gain greater therapeutic effects by reducing the cytotoxicity of the administered chemotherapeutic drug. SA, extracted from plants of the genus Oenanthe of the Umbelliferae family, is an original crude medicinal substance developed by Wang *et al* (Osaka University of Pharmaceutical Sciences, Osaka, Japan) (1); however, to our knowledge, no studies on its potential roles in cancer therapy have been published to date.

We previously reported the *in vitro* thermosensitization of human cancer cell lines by combined therapy with hyperthermia and the sesquiterpene lactone, parthenolide, an inhibitor of the transcription factor, NF-κB and further investigated the kinetics of apoptosis induction and the cell cycle distribution with regards to the transcription factor, NF-κB, and the proto-oncogene mitogen-activated protein (MAP) kinase (MAPK) signaling pathways (4-6). Cellular thermosensitivity was observed by combined treatment with parthenolide, an NF-κB inhibitor, and subsequent exposure to hyperthermia in A549 human non-small cell lung adenocarcinoma cells bearing the wild-type *p53* gene. Apoptosis was induced through the direct suppression of NF-κB in a p53-independent and heat-induced hsp72-independent manner via the NF-κB signaling pathway (4). We also reported that combination therapy with parthenolide administered prior to exposure to hyperthermia caused lethal damage to A549 cells by targeting the S phase; this effect correlated with the induction of apoptosis and the G_2/M arrest via the NF-κB cascade, and possibly occurred due to the blockade of NF-κB activation by the heat-induced hsp72 protein. It was concluded that parthenolide contributes to thermosensitization through other pathways related to NF-κB signaling or by crosstalk with other mediator genes (5). In another study, combination therapy with parthenolide and heating was carried out in the human androgen-independent prostate cancer cell lines, PC3 and DU145. We reported a higher number of apoptotic PC3 cells than DU145 cells, which was in agreement with the finding that the amount of damage to the cells (assessed by survival curves) was greater in the PC3 than in the DU145 cells (6).

Ras activates a number of pathways, of which MAPK has been the most studied. This cascade transmits signals downstream and results in the transcription of genes involved in cell growth or mitosis (7). The unusual activation of the Ras/Raf/MAPK signaling pathway is characteristic of human cancer; thus, the pathway has attracted attention as a potential target in anticancer therapy. Similar to hsp family members, known to be thermotolerance inducers, *ras* is known as a gene that causes thermoresistance in hyperthermia through various signaling cascades. The association of thermotolerance with the *ras* gene has been demonstrated, since thermotolerance or thermoresistance developed following the blockade of the activation of the Ras-cAMP or MAPK cascades (8-10). Among the variety of intracellular signaling pathways, the most relevant to the development of cell malignancy are the MAPK growth signaling cascade and the phosphoinositide 3-kinase (PI3K)-Akt survival signaling cascade. The MAPK pathway has been reported to be activated in various types of cancer, such as breast cancer, colon cancer, melanoma, lung cancer and prostate cancer, which indicates its involvement in tumor progression and metastasis (11-15). Three distinct groups of MAPKs have been identified in mammalian cells: the extracellular-regulated kinase (ERK), the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and p38 (16-19); they all act as mediators of signals from the cell surface to the nucleus (20). The SAPK/JNK signaling pathway also regulates cellular proliferation, apoptosis and tissue morphogenesis (21). Xia *et al* (22) reported opposing effects of the ERK and SAPK/JNK-p38 MAPKs on apoptosis; thus, the dynamic balance between the growth factor-activated ERK and the stress-activated SAPK/JNK-p38 pathways may be important for determining whether a cell survives or undergoes apoptosis (22). We previously reported that parthenolide in combination with hyperthermia activated the p-SAPK/JNK protein mainly through the abovementioned MAPK family signaling pathways (ERK1/2, SAPK/JNK and p38) in DU145 cells. However, our results further indicated that the induction of apoptosis and cell cycle arrest at the G_2/M phase mostly occurred via the SAPK/JNK pathway (6).

The MAPK pathway is activated by different extracellular stimuli and has distinct downstream targets; therefore its disruption can halt cancer progression by inhibiting tumor angiogenesis, proliferation, invasion and metastasis (23). In prostate cancer, a number of other pathways have been shown to be activated, including the PI3K/Akt and NF-κB signal transduction pathways, which have been associated with tumor development and progression (24,25). In the present study, we confirmed that SA and hyperthermia exert antitumor effects by inducing apoptosis and cell cycle arrest. The related mechanisms, involving the Ras/MAPK cascade in the development of thermotolerance through the regulation of ERK and SAPK/JNK, and the involvement of the PI3K/Akt signal transduction pathway in the inhibition of apoptosis, were also examined. Furthermore, we investigated the effects of combined therapy using SA and heating on the thermosensitization of DU145 human prostate cancer cells, an androgen-independent cell line, as well as the relevant mechanisms.

Materials and methods

Cells and culture medium. DU145 cells from a human androgen-independent prostate cancer cell line were provided by the Department of Urology of Kitasato University (Tokyo, Japan), courtesy of Dr Takefumi Sato. The cells were cultured in RPMI-1640 medium (Invitrogen Life Technologies, Grand Island, NY, USA) under standard conditions at 37˚C in a humidified incubator with 5% CO_2 in 95% air (26,27). RPMI-1640 medium was supplemented with 10% fetal bovine serum (ICN Biomedicas Inc., Aurora, OH, USA), 1% minimum essential medium (MEM) non-essential amino acid (NEAA) solution, 1% MEM vitamin solution (both from Invitrogen Life Technologies), 1% sodium pyruvate, and 1% of a penicillin and streptomycin mix (both from Nacalai Tesque Inc., Kyoto, Japan).

SA, hyperthermia and combined treatment. SA was dissolved in culture medium to a final concentration of 20.0 μM prior to use. Cells that adhered to the inner side of the bottom of culture flasks were exposed to SA by replacement with 6 ml of SA solution for various periods of time. The cells were then treated with 20.0 μM of SA at 37˚C for 4 h, which resulted in 50% lethal damage (LD_{50}). The SA solution was removed and the adhered cells were gently rinsed twice with culture medium containing 3% serum, then resupplied with 6 ml of RPMI-1640 medium at 37˚C. Hyperthermia was established by immersing culture flasks equipped with tightened screw tops in a temperature-regulated water bath (Advantec, model LF-480; Toyo Seisakusho Co., Ltd., 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 combined treatment, applications of SA and heating were sequentially performed, with cells first exposed to SA for 4 h, then rinsed twice with culture medium containing 3% serum, placed in RPMI-1640 medium, and finally subjected to hyperthermia. Kinetic assessments of the sensitivity of DU145 cells to SA and hyperthermia were carried out by colony formation 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.

Cells exposed to hyperthermia produce linear survival curvers. The relationship between the survival fraction S and the heating period T is then calculated as: $S = e^{-\alpha T}$, where S is the number of surviving cells, $-\alpha$ is the slope and T is the heating period. This relationship is more commonly represented as: $S = e^{-T/T_0}$ by defining T_0 as $1/\alpha$. When $T = T_0$, $S = e^{-1} = 0.037$. S_0, T_0 show the heating period required to reduce the experimental survival rate by 1/e. The paramater T_0 can then be used to characterize the thermosensitivity in this region of the curve.

The T_0 value, which was adopted as the criterion for cellular thermosensitivity and sensitivity to SA, was defined as 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 the semilogarithmic survival curves.

Apoptosis assay and cell cycle distribution. The kinetics of apoptosis induction, as well as the G_1 and G_2/M cell cycle arrest of DU145 cells following treatment with SA, hyperthermia and their combination were analyzed by flow cytometry. After 0, 12, 24 and 48 h of incubation at 37˚C following treatments, cells ($1x10^5$) were harvested by trypsinization, resuspended in culture medium, rinsed twice with ice-cold PBS(-), and fixed in ice-cold 70% ethanol, following the addition of PBS into the culture tubes at a rate of 3. 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., St. Louis, MO, USA) at room temperature for 30 min. The 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 was analyzed using a flow cytometer (Beckman Coulter, Inc., Fullerton, CA, USA). Immediately prior to analysis, cell suspensions were filtered through 40-μm diameter nylon meshes to remove cell aggregates and debris. Ten thousand events per determination were analyzed for each sample and the quantification of the cell cycle distribution was performed using software provided by the manufacturer. Cell cycle distribution based on DNA content is represented by a histogram.

Examination of ERK1/2, SAPK/JNK and Akt cascades by western blot analysis. Intracellular protein and the phosphorylation of ERK1/2, SAPK/JNK MAPK and Akt following SA application, heating, and their combination were examined in DU145 cells ($1x10^6$) by western blot analysis. Cells were harvested by trypsinization and resuspended in RPMI-1640 medium. After rinsing with ice-cold PBS twice, they were dissolved in RIPA lysis buffer containing a protease and a phosphatase inhibitor and were treated by freezing at -20˚C and thawing on ice 3 times. Cell lysates were centrifuged at 14,000 rpm at 4˚C for 10 min to remove cell debris. The supernatants were then diluted 2-fold with SDS-PAGE, and subjected to a block incubator at 95˚C for 3 min following stirring and vortex mixing and transformation. The protein contents of the supernatants were quantified using a protein assay kit (Bio-Rad Laboratories, Richmond, CA, USA). Aliquots of protein (10 μg) were subjected to western blot analysis using ERK1/2, SAPK/JNK, Akt and their phosphorylated antibodies (Cell Signaling Technology, Japan KK). Following electrophoresis on 10% polyacrylamide gels containing 0.1% solution dodecyl sulfate (SDS) and electrophoretic transfer onto Immobilon-P PVDF-membranes (Millipore Corp., Medford, MA, USA), the membranes were incubated with phosphorylated (p-)ERK1/2, p-SAPK/JNK and p-Akt antibodies. GAPDH antibody (Cell Signaling Technology) served as the loading control, as previously described (28).

Results

Thermosensitizing effects of SA with hyperthermia at 40-44˚C. The thermosensitizing effects of SA were investigated in DU145 cells based on semilogarithmic survival curves produced from the results of the single and the combined therapy with 20.0 μM of SA for 4 h, and hyperthermia at 40, 42, 43 and 44˚C (Figs. 1 and 2). The survival curve following the heating treatment at 40˚C for up to 6 h indicated a slight cytotoxicity, while synergistic antitumor effects were observed for the treatment with 20.0 μM SA for 4 h prior to heating at 40˚C (Fig. 1). Cellular lethal thermosensitivity was estimated based on the T_0 value (heating time required to reduce survival by 1/e), which was the reciprocal of the slope of the survival curve in the exponential phase. This value was 39.8 h for heating at 40˚C, and 9.55 h for sequential treatment with SA and heating at 40˚C (Table I). Similarly, the thermosensitivity of the cells increased with heating at 42˚C, and the T_0 value was 2.12 h for heating at 42˚C, and 1.29 h for sequential treatment with SA and heating at 42˚C. The survival of cells heated at 43˚C or 44˚C and of those receiving combined treatment with SA was estimated for up to a 40-min heating period (Fig. 2). The T_0 value was 20.2 min for heating at 43˚C and 12.4 min for the combined treatment with SA and heating at 43˚C, 14.4 min for heating at 44˚C, and 10.6 min for the combined treatment with SA and heating at 44˚C (Table I). Thus, the synergistic thermosensitizing effects were observed with the combination of thermotherapy at 40-44˚C and SA.

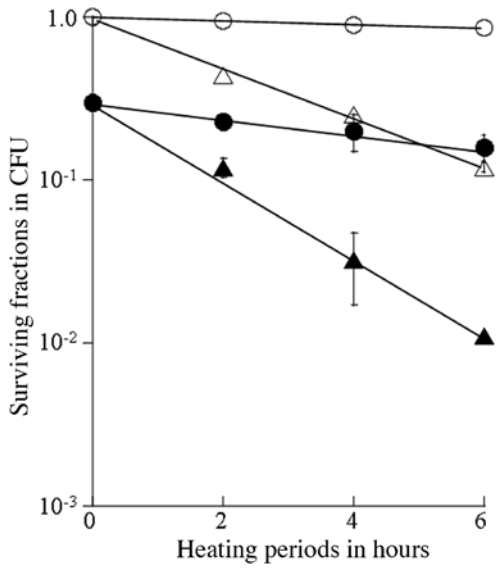

Figure 1. Thermosensitizing effects of sinodielide A (SA) combined with mild hyperthermia. Y-axis, survival fractions (SFs) in colony forming units (CFU) in log scale. X-axis, periods of heating at 40 or 42˚C in hours (h). Open circles, 40˚C hyperthermia; open triangles, 42˚C hyperthermia; closed circles, treatment with SA for 4 h followed by hyperthermia at 40˚C for graded periods; closed triangles, treatment with SA for 4 h followed by hyperthermia at 42˚C for graded periods. Symbols with vertical bars represent the means ± standard deviation (SD) obtained from at least 3 datasets. Symbols without error bars indicate that the SD is smaller than the symbol.

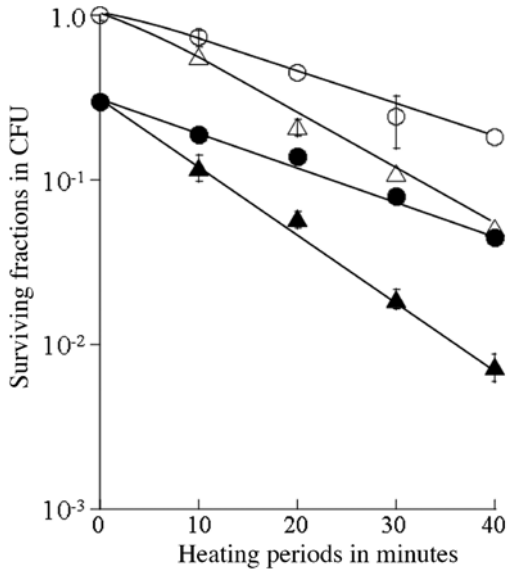

Figure 2. Thermosensitizing effects of sinodielide A (SA) combined with hyperthermia at high temperatures. Y-axis, survival fractions (SFs) in colony forming units (CFU) in log scale. X-axis, heating periods at 43 or 44˚C in minutes (min). Open circles, hyperthermia at 43˚C; open triangles, hyperthermia at 44˚C; Closed circles, tretment with SA for 4 h followed by hyperthermia at 43˚C for graded periods; closed triangles, treatment with SA for 4 h followed by hyperthermia at 44˚C for graded periods. Symbols with vertical bars represent the means ± standard deviation (SD) obtained from at least 3 data sets. Symbols without error bars indicate that the SD is smaller than the symbol.

Apoptosis assay and cell cycle distribution. Cell cycle distribution at 0, 12, 24 and 48 h after treatment with 20.0 μM SA for 4 h, 44˚C hyperthermia for 30 min, and their combination

Table I. Enhancement ratio based on T_0 values for survival of DU145 cells after subjection to hyperthermia and the combination of hyperthermia and SA.

Temp.	Hyperthermia T_{01}	SA and hyperthermia T_{02}	Enhancement ratio T_{01}/T_{02}
40˚C	39.81 h	9.55 h	4.16
42˚C	2.12 h	1.29 h	1.64
43˚C	20.2 min	12.4 min	1.63
44˚C	14.4 min	10.6 min	1.36

The thermal enhancement effects of the 4 levels of thermotherapy combined with SA were compared based on the T_0 values. The DU145 cells were treated with SA (20.0 μM) for 4 h, then subjected to hyperthermia at 40-44˚C in the combined treatment protocol. SA, sinodielide A. Temp., temperature.

was examined by flow cytometry, to examine the induction of apoptosis and cell cycle arrest in relation to the thermosensitivity of DU145 cells. Representative histograms showing the population distribution (based on DNA content as measured by PI) for the cell cycle phases (sub-G_1, G_1 and G_2/M) are shown in Fig. 3. The distribution of cells in the sub-G_1 (apoptotic cell population), the G_1 and G_2/M phases are expressed as a percentage (%) of the coefficient of variation (CV) values (Table II). The induction of apoptosis in the DU145 cells was estimated after 48 h by determining the number of cells in the sub-G_1 phase: 1.50±0.25% for the control, at 3.75±2.35% for SA treatment alone, at 4.20±1.30% for 44˚C heating treatment alone, and at 10.53±5.02% for the treatment combining SA and heating at 44˚C (Table II). With the combined treatment, SA significantly enhanced heat-induced apoptosis in the DU145 cells, which was estimated to be approximately 2.0-fold greater than when heating alone was applied. Fig. 4 shows the kinetics of apoptosis induction up to 48 h after treatment expressed as a percentage (%) of apoptotic cells at the sub-G_1 phase. The distribution (%) of DU145 cells at the G_2/M phase was significantly increased following heating at 44˚C (25.9±6.86%), and following the combined treatment of heating at 44˚C and SA (24.4±4.78%), as compared with the control (19.6±6.36%) (Table II). The distribution (%) of DU145 cells at the G_2/M phase was markedly increased at 24 h after heating at 44˚C, as well as after the combined treatment with SA (Fig. 3). Taken together, these results indicate that treatment with heating at 44˚C and the combination of heating at 44˚C and SA exert thermosensitizing effects by inducing cell cycle arrest at the G_2/M phase and apoptosis.

Western blot analysis of the MAPK cascade in response to treatment with SA and heating. We investigated the potential involvement of the Ras/Raf/MAPK and PI3K/Akt pathways in SA- and/or heat-induced apoptosis and cell cycle arrest in DU145 cells. The levels of constitutively activated proteins, as well as those of p-ERK1/2, p-SAPK/p-JNK and p-Akt in the MAPK cascade were assayed at 0, 12, 24 and 48 h following treatment with 20.0 μM of SA for 4 h, hyperthermia at 44˚C for 30 min, and their combination by western blot analysis

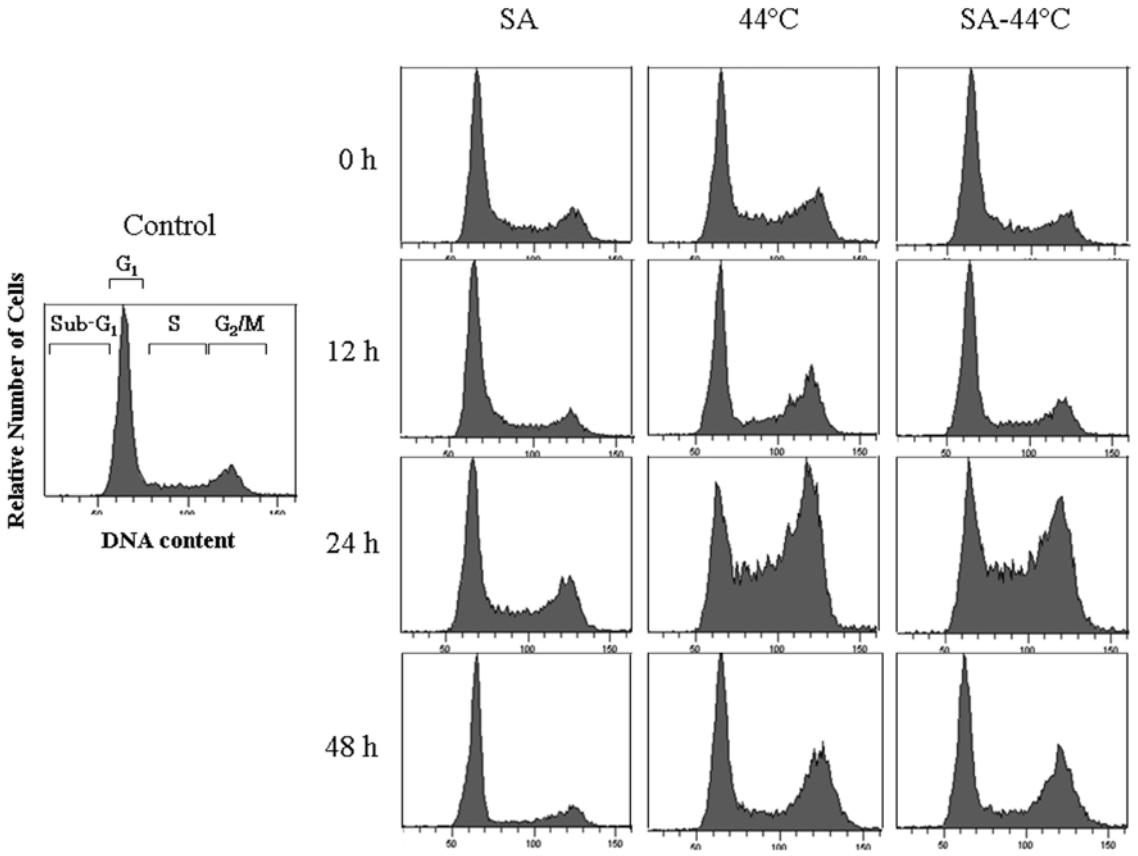

Figure 3. Determination, by flow cytometry, of the distribution (%) of DU145 cells in the different cell cycle phases analyzed (sub-G_1, G_1 and G_2/M phases) at 0, 12, 24 and 48 h of incubation at 37˚C following treatment with 20.0 μM sinodielide A (SA) at 37˚C for 4 h, hyperthermia at 44˚C for 30 min, and these treatments applied sequentially. Y-axis, relative number of cells. X-axis, DNA content expressed as the fluorescence intensity after propidium iodide (PI) staining. The panels show representative profiles of treated and untreated (control) cells based on data obtained from 2 or 3 independent experiments.

Table II. Distribution (%) of DU145 cell population in different phases of the cell cycle following treatment with SA, hyperthermia at 44˚C, and the combination of both.

Cell cycle phase	Interval periods (h)	Control	SA	Hyperthermia at 44˚C	SA + hyperthermia at 44˚C
Sub-G_1	0	1.5±0.25	2.23±0.45	2.17±0.66	3.10±0.40
	12		2.05±0.83	2.30±1.37	4.37±1.46
	24		3.03±1.97	3.02±1.77	4.40±0.80
	48		3.75±2.35	4.20±1.30	10.53±5.02
	Average		2.77±1.40	2.92±1.27	5.60±1.92
G_1	0	47.5±9.89	44.65±9.64	38.60±2.00	43.00±8.60
	12		48.72±3.46	41.47±5.20	45.77±5.24
	24		49.37±10.4	22.2±7.15	23.10±3.46
	48		57.60±8.74	30.02±3.00	30.82±6.70
	Average		50.08±8.06	33.07±4.34	35.67±6.00
G_2/M					
	0	19.6±6.36	23.7±6.82	22.2±5.32	21.6±6.47
	12		17.8±4.40	22.25±4.55	20.0±3.94
	24		19.4±3.99	34.6±9.70	28.6±3.34
	48		15.2±1.82	24.7±7.89	27.42±5.37
	Average		19.0±4.26	25.9±6.86	24.4±4.78

SA, sinodielide A.

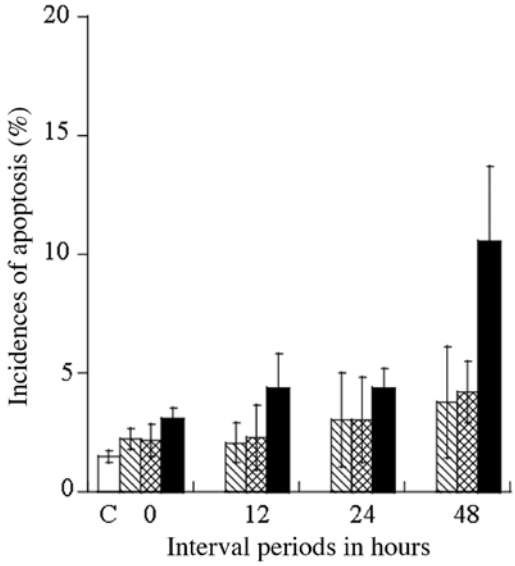

Figure 4. Quantification of apoptotic DU145 cell distribution at the sub-G_1 phase by flow cytometry. Y-axis, relative number of apoptotic cells expressed as a percentage of the total number of cells. X-axis, incubation-period at 37˚C, expressed in h after treatment. Columns from left represent: blank, untreated control (C); slanting stripes, treatment with 20.0 μM sinodielide A (SA) for 4 h; checkers, heating at 44˚C for 30 min; black, combined treatment.

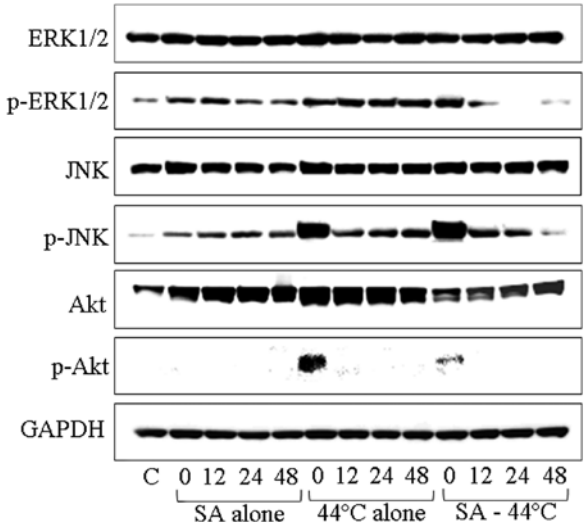

Figure 5. Western blot analysis of cellular amounts of extracellular-regulated kinase (ERK)1/2, phosphorylated (p-)ERK1/2, stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), p-SAPK/JNK, Akt and p-Akt in DU145 cells following treatment with 20.0 μM sinodielide A (SA) for 4 h, hyperthermia at 44˚C for 30 min, and the combination of these treatments. The numbers 0, 12, 24 and 48 on each well denote intervals of incubation at 37˚C, expressed in h after treatment, while 'C' represents the untreated control.

(Fig. 5). ERK1/2 levels were not increased in the DU145 cells by any of the treatments, while the phosphorylation of ERK1/2 was suppressed following combined treatment with SA and heating, since it appeared to localize in the cell nuclei in smaller quantities (Fig. 5). Furthermore, the SAPK/JNK levels were not increased after any of the treatments. However, the level of p-SAPK/p-JNK was markedly increased immediately after treatment with heating at 44˚C and after the combined treatment (Fig. 5). In addition, the levels of Akt markedly increased at 12, 24 and 48 h after treatment with SA and increased even further at 0, 12 and 24 h following treatment with heating at 44˚C, whereas the combination of SA and heating resulted in a slight increase at 48 h after treatment; this increase was less pronounced than the one observed with heating alone. Finally, the level of p-Akt/p-PKB was increased only immediately after treatment with heating at 44˚C.

Discussion

If thermal sensitizers are found to be effective in clinical anticancer thermotherapy by enhancing thermosensitivity or by significantly reducing thermotolerance without any side-effects, then they can be used in anticancer therapy for certain patients. We previously studied various chemicals, such as parthenolide, adriamycin (doxorubicin), bleomycin, cisplatin and amrubicin and its metabolite, amrubicinol, with regards to their ability to modify the effects of hyperthermia at the kinetic and molecular level (4-6,26-29). Cellular thermosensitivity was acquired through the combined treatment with parthenolide, an NF-κB inhibitor, and hyperthermia in A549 human non-small cell lung adenocarcinoma cells bearing the wild-type *p53* gene. The mechanisms underlying this effect appeared to involve the induction of apoptosis by the direct suppression of NF-κB in a p53-independent and heat-induced hsp72-independent manner via the NF-κB signaling pathway (4). Combined therapy using parthenolide and hyperthermia blocked the re-activation of NF-κB by the heat-induced hsp72 protein, and we suggested that parthenolide contributed to thermosensitization via another pathway related to NF-κB signaling or by crosstalk with another mediator gene (5). In PC3 cells, parthenolide in combination with hyperthermia gradually activated all phosphorylation cascades, with the most prominent increase observed for p-ERK1/2 and p-p38, which were associated with the induction of apoptosis or G_2/M cell cycle arrest mainly via the ERK1/2 and p38 cascades (6). Parthenolide in combination with hyperthermia activated p-p38 and p-SAPK/p-JNK in DU145 cells, an effect associated with the induction of apoptosis and G_2/M cell cycle arrest mainly via the SAPK/JNK cascades. The contrasting results between the two cell lines may be due to the fact that PC3 cells are null for the *p53* gene, while DU145 cells possess a mutant copy of *p53*. Furthermore, parthenolide- and heat-induced apoptosis and G_2/M cell cycle arrest in PC3 cells that do not have the *p53* gene occurred via the ERK1/2 and p38 MAPK signaling cascades with the upregulation of NF-κB, irrespective of p53, while the same effects in DU145 cells possessing a mutant *p53* copy may have been caused by a slight *p53*-dependent thermoresistance. In the present study, we investigated the medicinal herbal compound, SA, which has been shown to have few side-effects (1), for use in thermochemotherapy; the long-term aim was to identify molecules exerting therapeutic effects, while overcoming problems associated with thermotolerance and/or drug resistance. In addition, we examined the mechanisms involved in the *in vitro* interaction of hyperthermia and chemotherapy at the kinetic and molecular level, using DU145 human prostate cancer cells.

Thermosensitizing effects of SA combined with heating at 40-44˚C. The survival curve for heating at 40˚C indicated a slight heat-induced cytotoxicity for up to 6 h, while treatment with 20.0 μM SA for 4 h prior to heating at 40˚C enhanced the antitumor effects in a synergistic manner. The T_0 value was 39.8 h for heating treatment at 40˚C and 9.55 h for the sequential combination of SA and heating at 40˚C (Table I). Similarly, the thermosensitizing effects became more prominent with heating at 42˚C, showing a T_0 value of 2.12 h, while T_0 was 1.29 h for the sequential treatment with SA and heating at 42˚C. Moreover, the T_0 values for heating at higher temperatures were 20.2 min for 43˚C and 14.4 min for 44˚C, while they were 12.4 min for the combination of SA and heating at 43˚C, and 10.6 min for SA and heating at 44˚C. The thermal enhancement ratios for SA at 40, 42, 43 and 44˚C were 4.16, 1.64, 1.63 and 1.36, respectively. These results demonstrated that the thermosensitizing effects were more pronounced with the combination therapy with SA and lower temperatures; in particular, the use of mild hyperthermia and SA reduced the heating time required to obtain effects equal to those induced by hyperthermia alone.

Apoptosis assay and cell cycle distribution. A number of studies on gene and signaling pathways related to the induction of apoptosis have been presented. Mayo *et al* (30) reported that the activation of NF-κB suppressed the induction of p53-independent apoptosis, while Kalra *et al* (31) found that apoptosis in prostate cancer cells was mediated through two related pathways; the upregulation of *p53* and the downregulation of NF-κB. Hyperthermia-induced apoptosis has also been found to be mediated by caspase-3 (32). Furthermore, other studies have demonstrated that the inhibition of NF-κB induces apoptosis and cell cycle arrest in the G_2/M phase through various signaling pathways. In addition, the inhibition of the radiation-induced activation of NF-κB in prostate cancer cells has been shown to promote apoptosis and G_2/M cell cycle arrest, which correlates with increased p21/WAF1/Cip1 and decreased cyclin B1 expression (33). In another study, the viability of HeLa cells was reduced by inducing cell cycle arrest at the G_2/M phase and mitochondrial apoptosis through the *p53*-dependent expression of proteins of the Bcl-2 family (34). In addition, the pathway for NF-κB/caspase activation was shown to be independent of the NF-κB/cell cycle pathway, and the events downstream of the NF-κB/caspase-9 cascade have been shown to lead to apoptosis (35). In the present study, using flow cytometry, we examined the kinetics of apoptosis at various periods of time following exposure to SA (20.0 μM) for 4 h, hyperthermia at 44˚C for 30 min, and the combination of these treatments using DU145 cells (Fig. 3). The distribution of the cell populations at the sub-G_1 (apoptotic cell population), G_1, and G_2/M phases after these treatments is shown in Table II. The induction of apoptosis is indicated by the average percentage of cells at the sub-G_1 phase at 48 h and was estimated at 1.50±0.25% for the control, 2.77±1.40% for the SA-treated cells, 2.92±1.27% for the 44˚C heat-treated cells, and 5.60±1.92% for the combination of these treatments (Table II). When used in combination with heating, SA markedly enhanced heat-induced apoptosis in DU145 cells by approximately 1.9-fold, as compared with heating treatment alone. The thermosensitizing effects, estimated by examining the induction of apoptosis in DU145 cells (percentage of cells in the sub-G_1, G_1 and G_2/M phase) became more prominent with time. The DNA content at the G_2/M and G_1 fractions in the flow cytometry distributions indicated the amount of cells in cell cycle arrest. The greatest increase was observed for cells in G_1 arrest following treatment with SA (50.08±8.06%), while the percentage of cells in G_2/M arrest was increased by both heating at 44˚C and the combined treatment to 25.9±6.86 and 24.4±4.78%, respectively, as compared with the control (Table II). In addition, the relative amount of DNA content in the G_2/M cell fraction after heating at 44˚C and the combined treatment with SA was relatively high at 24 h (Fig. 3).

Examination of MAPK and Akt cascades by western blot analysis. *Ras* regulates multiple downstream effector pathways, such as Raf/MAP/ERK, PI3K/Akt and JNK/p38 (36-41). The Ras/Raf/MAPK cascade promotes mitogen activation and cell growth via the EGFR signaling pathway, which mainly participates in proliferation, inhibition of apoptosis, infiltration and metastasis (42,43). Among a number of intracellular signal transduction pathways, the most relevant to cancer development are the MAPK/ERK (growth signaling) and PI3K/Akt (survival signaling) cascades regulating the downstream gene, *ras*. A previous study demonstrated that DU145 cells possess mutant the *ras* gene (44). In this study, we examined the constitutional and phosphorylated activation of ERK1/2, JNK/SAPK and Akt in cascades related to the *ras* signaling pathway, regulating cell cycle check points and transcription factors, in order to investigate the mechanisms underlying the thermosensitizing effects of the combination of SA and hyperthermia in DU145 cells possessing mutant *ras* copies. The dimerization product of p-ERK1 and p-ERK2, ERK1/2, translocates from the cytoplasm to the nucleus to activate the transcription factor, NF-κB (45,46). However, another study suggested that ERK induces neuronal apoptosis (47). ERK1/2 in DU145 cells was not increased following treatment with SA alone, heating at 44˚C, or the combination of both, while ERK1/2 phosphorylation was suppressed by treatment with the combination of SA and heating and was localized in small quantities in the nucleus. By contrast, the SAPK/JNK level was increased by each of these treatments, with the most prominent increase of its phosphorylated form occurring immediately after treatment with heating at 44˚C and after the combined treatment with SA (Fig. 5). The JNK signaling pathway induces apoptosis through diverse mediators (48-51). We found that the Akt level was markedly increased at 12, 24 and 48 h following treatment with SA, and at 0, 12 and 24 h following heating at 44˚C. By contrast, the combination of these treatments only slightly increased the Akt level for up to 48 h after treatment. Furthermore, p-Akt levels were increased only immediately after heating at 44˚C. Thus, SA further contributed to thermosensitization by inducing apoptosis and G_2/M arrest by inhibiting the phosphorylation of Akt. The PI3K/Akt pathway regulates apoptosis, a process where kinetics are either pro- or anti-apoptotic, depending on the type of stimuli and circumstances (52,53). Numerous studies on the PI3K/Akt pathway have indicated that its inhibition can inhibit the proliferation of cancer cells (54-57). We conclude that SA exerts its thermosensitizing effects on

DU145 cells by inhibiting the activation of the ERK1/2 and PI3K/Akt signaling pathways and promoting apoptosis via the JNK signaling cascade.

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