Molecular Mechanisms and Gene Regulation of Melphalan- and Hyperthermia-induced Apoptosis in Ewing Sarcoma Cells

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Abstract. *The prognosis of high-risk Ewing tumours (HR-ET) remains poor. Melphalan-containing chemotherapy regimens are commonly applied for HR-ET patients. Moreover, melphalan (Mel) is a promising agent in thermochemotherapy. Therefore, we investigated the single effects, the synergism and the gene regulation of Mel and hyperthermia (HT) in an ET cell line (RD-ES). Dose-dependent cytotoxicity by Mel was demonstrated, which was enhanced by the concomitant application of HT (42˚C for 2 h). Mel, HT and their combination caused a significant activation of caspase-3. Using the pan-caspase inhibitor z-VAD-fmk, we demonstrated that both stimuli mediated predominantly caspase-dependent cytotoxicity. With cDNA array analysis, 20 out of 198 apoptosis-related genes were identified to be differentially expressed by Mel and/or HT. Although a significant enhancement of three selected genes could not be proven at the protein level in subsequent experiments, this study gives insight into the complex molecular and genetic response of tumour cells to cytotoxic stimulation.*

Ewing tumours (ET) are the second most frequent bone tumours in childhood and adolescence, with an annual incidence of about 3 cases per million (43). Prognosis depends on tumour volume, tumour site, histological response to chemotherapy and, most importantly, the presence of metastases at the time of diagnosis. In particular, patients with metastases to bone or bone marrow still have a poor prognosis (43). These patients might benefit from intensification of conventional therapy, for instance by thermochemotherapy. It has been shown that melphalan (Mel) is one of the most promising agents in thermochemotherapy, as its anticancer effect was effectively enhanced by hyperthermia (HT) in many experimental and clinical trials (16, 18, 37, 39, 40, 47, 51-53). Moreover, Mel is commonly applied in high-dose chemotherapy for the treatment of high-risk ET (4, 29, 45). However, there is still no report of a combined use of Mel and HT in the treatment of patients with ET, although HT has already been reported to enhance the effect of other cytotoxic drugs in ET, such as cisplatin (3, 7, 8).

In different tumour models, Mel induced cytotoxic effects concomitant with an activation of caspase-3 (5, 14, 25). Moreover, numerous previous studies have shown an involvement of caspases in HT-induced apoptosis (15, 17, 19, 20, 22, 23, 34-36, 54). However, there are only few reports concerning the underlying mechanisms in response to the cytotoxic stimulation with Mel or HT in tumour cells.

Hence, in the present study we investigated the single effects, the synergism and the gene regulation of Mel and HT in a human ET cell line (RD-ES) to answer the following questions: a)Do RD-ES cells respond to Mel or heat stimulation? b)Does HT enhance Mel-induced cytotoxicity? c)Which molecular mechanisms are involved in the cytotoxic effects of Mel and HT? d) Can a synergistic effect of Mel and HT be explained by a recruitment of different molecular pathways by either stimulus?

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Materials and Methods

Cell line and culture conditions. The human Ewing tumour cell line RD-ES, which was derived from a primary Ewing's sarcoma of a 19-year-old Caucasian male in 1984, was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The RD-ES cells were maintained in RPMI-1640 medium (Gibco BRL, Karlsruhe, Germany) supplemented with 15% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin (all additives from Biochrom Seromed, Berlin, Germany), and were cultured in a humidified atmosphere at 37˚C and 5% CO_2.

Cytotoxic stimulation. Melphalan was provided as stock solution (1 mg/ml dissolved in 0.3% NaCl) by the central pharmacy of the University of Leipzig Medical Center, Germany. RD-ES cells were

plated at a density of 1×10^6/ml and stimulated with different concentrations of Mel (0.1-90 μg/ml) or no drug at all. After incubation at 37˚C and 5% CO_2 for 1 h, cell culture dishes were wrapped in laboratory film (Parafilm; American National Can, Chicago, IL, USA) and immersed in a waterbath for 2 h at 37˚C (control) or 42˚C (hyperthermia, HT), respectively. Thereafter, the culture plates were unwrapped and re-exposed to a humidified atmosphere at 37˚C and 5% CO_2.

Determination of apoptosis and cell death. Apoptosis and cell death were quantified using an Annexin V-FITC staining kit (Immunotech, Coulter Company, Marseilles, France) and flow cytometry analysis. After stimulation, the cells were harvested with trypsin-EDTA (Biochrom Seromed) and stained with Annexin V-FITC (AV; 25 μg/ml) and propidium iodide (PI; 250 μg/ml), according to the manufacturer's guidelines. The fraction of vital cells was determined by flow cytometry (Epics XL; Beckman Coulter, Krefeld, Germany) as the double negative fraction that stained neither positive for AV nor for PI. For evaluation of the results, the software Expo 32 (Beckman Coulter) was used. The results were expressed as specific cytotoxic rates, defined as follows: *Cytotoxicity = 100% – % vital cells (AV–/PI–); Specific cytotoxicity = cytotoxicity (stimulated sample) – cytotoxicity (unstimulated control)*.

For kinetic analyses, cytotoxicity was measured by performing AV/PI assay at the following time intervals: immediately after water bath incubation (t_1), after an additional 2 h (t_2), 6 h (t_3), 12 h (t_4) and after a total of 24 h (t_5) of culture duration. Cytotoxicity rates at the different time points were expressed as specific cytotoxicity per hour, calculated as follows: *Specific cytotoxicity (interval t_n-t_{n+1}) = (specific cytotoxicity (t_{n+1}) – specific cytotoxicity (t_n)) / h (interval duration)*.

Determination of caspase-3 activity. Activation of caspase-3 was measured by using an FITC-conjugated anti-active caspase-3 monoclonal antibody (Clone C92-605; BD Biosciences PharMingen, San Diego, CA, USA). RD-ES cells were stimulated with Mel (10 μg/ml) and/or HT (42˚C/2 h) for 24 h. The cells were harvested, washed and processed using the Cytofix/Cytoperm Kit (BD Biosciences PharMingen, San Diego, CA, USA) according to the manufacturer's instructions. Thereafter, the cells were stained with anti-active caspase-3 monoclonal Ab for 60 min at room temperature in the dark and analysed by flow cytometry.

General caspase inhibitor assay. The general caspase inhibitor z-VAD-fmk (R&D Systems, Wiesbaden, Germany) was reconstituted in dimethyl sulfoxide (DMSO) to yield a 20 mM stock solution. Z-VAD fmk was used at a concentration of 100 μM. Cells were preincubated with z-VAD-fmk or an equal concentration of DMSO as a solvent control for 1 h. The cultures were then stimulated with Mel (10 μg/ml) and/or HT as described above. AV/PI staining was carried out 24 h after addition of the drug. Results were expressed in cytotoxicity rates considering the following definitions: *Total specific cytotoxicity = cytotoxicity in the presence of the stimulus and DMSO – cytotoxicity in the presence of culture medium and DMSO; Specific caspase-independent cytotoxicity = cytotoxicity in the presence of the stimulus and z-VAD-fmk – cytotoxicity in the presence of culture medium and z-VAD-fmk; Specific caspase-dependent cytotoxicity = total specific cytotoxicity – specific caspase-independent cytotoxicity*.

Isolation of RNA. RD-ES cells were harvested by trypsinization 6 h after stimulation with Mel (10 μg/ml) and/or HT. Total RNA was then extracted using the Invisorb® Spin Cell RNA Mini Kit (Invitek GmbH, Berlin, Germany) according to the manufacturer's instructions. The integrity of the RNA and the absence of DNA contamination were verified by agarose gel electrophoresis. RNA concentration and purity were determined photometrically by calculating the ratio of optical density at 260 and 280 nm.

cDNA array analysis. Gene expression was analysed with the Human Apoptosis Expression Array (R&D Systems, Minneapolis, USA), which contains 198 apoptosis-related gene products, 8 positive control housekeeping genes and 6 negative controls, all spotted as duplicates. Briefly, 2 μg of RNA from each sample were converted into ^{32}P-labelled single-strand cDNA by reverse transcription using cDNA labelling and hybridisation kit and apoptosis-specific primers (R&D Systems, Minneapolis, USA), and incorporating [α-^{32}P]-deoxycytidine-triphosphate (Amersham, Piscataway, USA). The labelled cDNA was purified with Spin Columns (R&D Systems), and hybridised to the filter array overnight at 65˚C. Thereafter, the array membranes were washed, exposed to a phosphor imaging plate (Fuji Photo Film Company, Kanagawa, Japan), scanned with a Phosphor Imager Analyser (FLA 3000; Fujifilm, Kanagawa, Japan) and analysed by AIDA Software (Raytest, Straubenhardt, Germany). For each array, the absolute signals of the different genes were normalized to an average of the expression of the 8 spotted housekeeping genes. The relative signals of the stimulated samples were then compared with the corresponding normalised signals of an unstimulated sample as a control.

Inhibition of the interaction of CD137 and CD137L. RD-ES cells were preincubated with 20 μg/ml of the nonstimulatory monoclonal anti-CD137 Ab clone BBK 2 (Neomarkers, Fremont, CA, USA) or IgG-1 (R&D Systems, Wiesbaden, Germany) as a control for 1 h before stimulation with Mel (10 μg/ml) and/or HT. Cytotoxicity was measured 6 h post stimulation using AV/PI staining (see above).

Analysis of the protein expression of CD137L. Cell surface expression of CD137L was determined immediately after stimulation (0 h) with Mel (10 μg/ml) and HT and after 1, 2, 4, 6 and 12 h of additional cell culture. Cells were harvested by trypsinization and washed 3 times in wash buffer [phosphate buffered saline (PBS), 2% FCS]. The cells were then resuspended in 50 μl wash buffer and stained for 30 min with either 20 μl FITC-labelled monoclonal anti-CD137L Ab [clone 5F4, (50 μg/ml); MBL, Nagoya, Japan] or 20 μl mouse-IgG-1-FITC (Immunotech) as a control. The cells were washed once and analysed by flow cytometry.

Western blot analysis of DRAK2. To investigate the protein expression of DRAK2 in the RD-ES cells, the nuclear protein was isolated 6 h after stimulation with Mel (10 μg/ml) and/or HT. The cells were harvested without the use of trypsin, washed twice in ice-cold PBS, and collected by centrifugation. The cells were resuspended in 1 ml lysis buffer [10 mM Hepes-KOH (pH 7.6), 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mM ethylene diamine tetraacetic acid (EDTA), 15 mM KCl, 2 mM $MgCl_2$], and incubated for 10 min on ice. After centrifugation at 700×g for 5 min, the supernatant (cytoplasm) was discarded. The pellet (nuclei) was resuspended in 50 μl buffer C [20 mM Hepes-KOH (pH 7.9) 0.5 mM DTT, 0.5 mM PMSF, 0.2 mM EDTA, 420

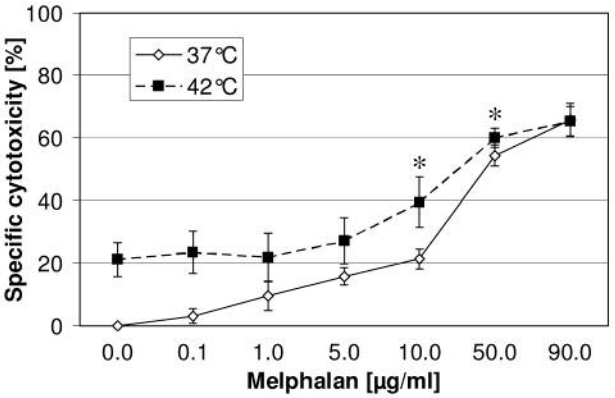

Figure 1. *Mel and HT induce cell death in RD-ES cells, the combination of both results in dose-dependent enhancement of cytotoxicity. RD-ES cells were cultured with the indicated concentrations of Mel (0-90 µg/ml) and/or HT (42˚C/2 h) as described in detail in the Materials and Methods section. Cell death was determined by AV/PI staining and flow cytometry after 24 h of culture. Specific cytotoxicity was calculated as defined in the Materials and Methods section. Mean and SEM values of 6 independent experiments are shown. Significant enhancement of cytotoxicity ($p<0.05$) by combination of Mel and HT compared to the effect of each single stimulus is indicated by *.*

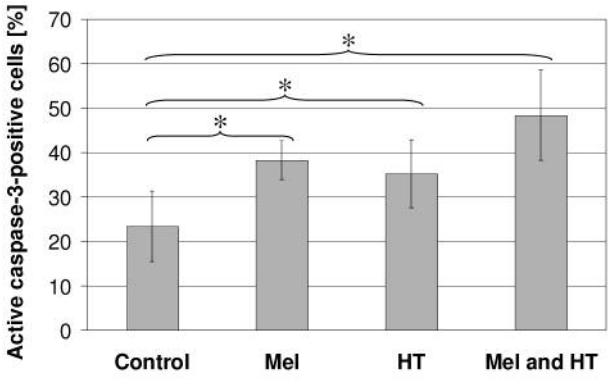

Figure 2. *Activation of caspase-3 by Mel and HT. RD-ES cells were stimulated with Mel (10 µg/ml) and/or HT. Induction of caspase-3 activity was determined after 24 h of culture under standard conditions using an FITC-conjugated anti-active caspase-3 monoclonal antibody and flow cytometry analysis. Mean and SEM values of 3 independent experiments are shown. A significant increase of active-caspase-3-positive cells compared to control cells ($p<0.05$) is indicated by *.*

mM NaCl, 25% (v/v) glycerin] and incubated on ice for 15 min. The protein levels in supernatants obtained after centrifugation (10,000×g for 10 min) were quantified with a BioRad protein assay kit (BioRad, CA, USA) according to the manufacturer's instructions. Aliquots of the proteins (50 µg) were subjected to Western blot analysis for DRAK2. After electrophoresis on 10% SDS polyacrylamide gels, the proteins were transferred electrophoretically onto PVDF membranes (BioRad). The membranes were incubated with 1:500 rabbit polyclonal anti-DRAK2 Ab (Abcam, Cambridge, UK) for 90 min at room temperature. Blots were then washed 3 times for 10 min in Tris buffered saline (TBS) and incubated with 1:40,000 secondary horse radish peroxidase-conjugated goat anti-rabbit IgG (Fc) (R&D Systems) for 1 h at room temperature. After washing off excess secondary Ab, the signals were detected with SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce Biotechnology, Inc., Rockford, IL, USA) and Hyperfilm ECL (Amersham Biosciences, Buckinghamshire, UK).

Detection of TRAIL-R2. Cell surface expression of TRAIL-R2 was detected 6 h after stimulation of RD-ES cells with Mel (10 µg/ml) or HT. Cells were harvested by trypsinization and stained with 5 µg/ml FITC-labelled monoclonal mouse-anti-human TRAIL-R2 Ab (clone HS201; R&D Systems) for 10 min at 4˚C or mouse-IgG-1-FITC (Immunotech) as a control. The cells were then analysed by flow cytometry. The portion of TRAIL-R2 positive cells was determined by subtracting the percentage of fluorescing cells using the staining control from the percentage of cells stained with the anti-TRAIL-R2 Ab.

Statistical analysis. Results are presented as means±standard error of the mean (SEM). For statistical analysis, Student's *t*-test for paired samples was used, applying a two-tailed Student's *t*-test for the cDNA array analysis and a one-tailed Student's *t*-test for all other experiments. A value of $p<0.05$ was considered to be statistically significant.

Results

Dose-response relation of Mel and HT in RD-ES cells. Mel induced apoptosis and cell death in the Ewing's sarcoma cell line RD-ES in a dose-dependent manner (Figure 1), mediating significantly enhanced cytotoxicity at a drug concentration of 1.0 µg/ml and above. Isolated heat application led to a significant reduction of the vital cell fraction of 21.3±5.4%. The combination of Mel with HT resulted in a significant increase of cell death at the concentrations 10 and 50 µg/ml Mel compared to either single stimulus. Since the enhancement of the cytotoxic effect was almost additive at a drug concentration of 10 µg/ml, for all further experiments this concentration was used. Mel-specific cytotoxicity was elevated to 39.5±8.0% under hyperthermic conditions compared to 21.4±3.1% at normothermia at this particular drug concentration.

Activation of caspase-3. In RD-ES cells the level of activated caspase-3 was elevated by both Mel and HT application, compared to the unstimulated control. Maximum levels were observed with the combination of both stimuli, yet this caspase activation was not significantly higher than with either single stimulus (Figure 2).

Caspase-dependent and -independent mechanisms. To investigate whether caspases were required for the induction of cell death by Mel and/or HT, caspase activation was blocked with the general caspase inhibitor z-VAD-fmk. Thereby caspase-dependent and caspase-independent cytotoxicity could be distinguished. As shown in Figure 3, Mel- and HT-induced cell death was completely caspase-

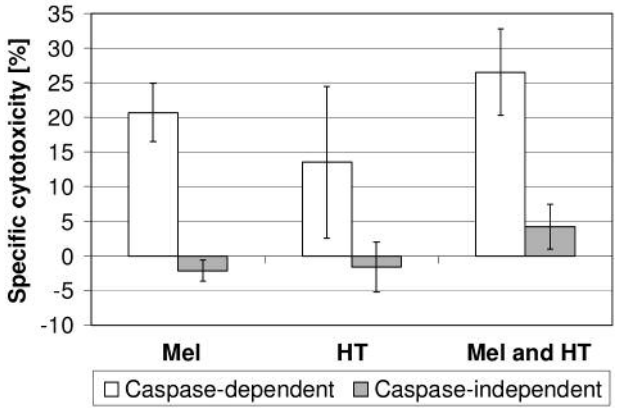

Figure 3. *Role of caspase-activation in Mel- and HT-induced cytotoxicity. Cell cultures were preincubated with the general caspase inhibitor z-VAD-fmk (100 μM) or equal volumes of DMSO 1 h before application of Mel (10 μg/ml) and/or HT. Cytotoxicity was measured by AV/PI staining and flow cytometry 24 h after stimulation. Results are expressed as caspase-dependent and caspase-independent cytotoxicity calculated as described in the Materials and Methods section. Mean and SEM values of 4 independent experiments are shown.*

dependent in the RD-ES cells. The combined stimulus resulted in the occurrence of a marginal portion of caspase-independent cytotoxicity, while the majority of the cytotoxicity remained caspase-dependent.

Kinetics of HT- and Mel-induced cell death. First significant cytotoxic effects of Mel- and/or HT-induced cytotoxicity in RD-ES cells appeared early, *i.e.* at 2 h after stimulation. Interestingly, Mel showed a different time-course of cell death induction compared to HT (Figure 4). Mel continuously induced apoptosis throughout the entire experiment of 24 h duration at a nearly constant level of about 0.4-0.9% per h, whereas heat had a maximum effect between 2 and 6 h after stimulation, with a cytotoxic rate of 2.4±0.9% per h. After 12 h, heat mediated no further cytotoxicity. The combined application of Mel and HT reflected the time kinetics of both stimuli with an early maximum and a persistent level of cytotoxicity throughout the 24 hours of observation. However, between 2 and 6 h post stimulation, the combination of Mel and HT achieved a superadditive effect (cytotoxic rate of 4.6±0.5% per hour) compared to each stimulus given alone.

Effects of Mel and/or HT on the expression of apoptosis-related genes. To investigate the differential regulation of gene expression by Mel and/or HT, a commercial cDNA array from R&D Systems was used, featuring 198 apoptosis-related genes, including pro- and antiapoptotic factors, cell cycle regulators, caspases, signal transduction factors, as well as cytokines and their receptors. Total RNA was isolated 6 h after stimulation of the RD-ES cells with Mel and/or HT, complementary DNA was produced and hybridised with the cDNA array membranes. The intensity of each duplicate spot was measured and normalized to the average expression of concomitantly hybridised housekeeping genes.

To decide whether there was substantial up- or down-regulation in the gene expression in RD-ES cells following treatment, a threshold of a twofold difference was implemented. This expression difference had to be significant throughout three independent experiments. Genes with a general expression level below 2.0 AE were excluded from analysis. According to these definitions, 20 out of 198 genes were identified to be differentially expressed after the treatment with Mel and/or HT (Table I). The single application of Mel resulted in enhanced mRNA expression of 3 genes (*caspase-3, p21* and *TRAIL-R4*), whereas the expression of 4 genes was suppressed (*XIAP, RB1, IGF-I R* and *IL-1 R AcP*). HT induced the transcription of 11 genes (*eNOS, TFAR15, caspase-8, bcl-10, FAN, par-4, PTEN, Fas, TRAIL-R1, TRAIL-R2* and *TRAIL-R4*). The expression of *bcl-10* was also significantly enhanced by the combined stimulation with Mel and HT. Other genes that were synergistically stimulated by Mel and HT were *4-1BBL* (CD137L) and *DRAK2*. The gene for *PAK1B* was the only gene which was down-regulated by the combined stimulus.

Inhibition of the interaction of CD137 and CD137L. CD137L was reported to mediate a pro-apoptotic effect in the ligand-bearing cells when stimulated in the context of reverse signalling (38, 50). The binding of CD137L to its receptor was blocked with a non-stimulatory Ab (BBK-2) to investigate the role of CD137L in mediating cytotoxicity through Mel in combination with HT. The inhibition of the CD137 and CD137L interaction had no effect on the amount of cell death induced by Mel and HT (Figure 5). This finding was unexpected and prompted us to examine whether the membrane expression of CD137L at all resembled the mRNA expression changes which were seen after the stimulation with Mel and HT. By using a FITC-labelled anti-CD137L Ab and flow cytometry, we demonstrated that neither unstimulated RD-ES cells nor Mel- and HT-treated cells exhibited CD137L protein on the cell surface (data not shown).

DRAK2 expression in RD-ES cells. To investigate the protein expression of *DRAK2*, another pro-apoptotic gene whose mRNA expression was up-regulated by the combined stimulus of Mel and HT, nuclear protein was isolated from the RD-ES cells and Western blot analysis was performed. DRAK2 protein could be detected in the RD-ES cells under all culture conditions, *i.e.* in stimulated as well as in unstimulated cells (Figure 6). A major enhancement of the protein expression after treatment with Mel and HT was not recorded by this semi-quantitative method.

TRAIL-R2 expression. Finally, the membrane expression of a third gene, *TRAIL-R2*, was investigated, since *TRAIL-R2* showed a high basal expression in the control culture and was enhanced by HT to nearly threefold. Likewise, a great percentage of RD-ES cells in the unstimulated control culture expressed the pro-apoptotic TRAIL-R2 receptor on the cell membrane. Heat stimulation seemed to further enhance membrane expression, but only to a slight extent, while Mel had no significant effect on the protein expression of TRAIL-R2 (Figure 7).

Discussion

In the present investigation, Mel induced cell death in the Ewing's sarcoma cell line RD-ES in a dose-dependent manner. Mel mediated markedly enhanced cytotoxicity in a concentration range (5-10 μg/ml) which is achieved in the plasma of patients treated with Mel (44). Thus, RD-ES cells can be regarded as Mel-sensitive. Generally, Ewing tumours respond well to treatment with Mel, which is frequently used in high-dose chemotherapy regimens for patients with ET (4, 29, 45). In the present study, HT (42˚C/2 h) also induced remarkable cytotoxicity in the RD-ES cells. Similar results were published by Debes and co-workers, who investigated the effect of HT on different ET cell lines; all tested cell lines responded to heat treatment (7, 8). Furthermore, it was shown in the present study that HT enhanced the cytotoxic effect of Mel when applicated simultaneously with the drug. Although an enhancement of Mel-induced cytotoxicity under hyperthermic conditions has been shown in many tumour models (18, 39, 40, 51-53), to date there has not been any report of a synergistic effect of Mel and HT in ET cells.

By further investigating the cytotoxic effect in the context of apoptosis the role of caspases became clear for both Mel and HT. Both stimuli induced activation of caspase-3, which is one of the key effector caspases in apoptosis. By inhibiting caspase activation with a general caspase inhibitor, it was shown that the cytotoxicity of both stimuli was almost completely caspase-dependent. In former studies, we and others have shown the important role of caspases for the cytotoxic effect of heat in various tumour cells. Kobayashi *et al*. demonstrated heat-induced apoptosis by caspase-3 activation in HeLa cells. Administration of a caspase-3 inhibitor diminished the heat-induced apoptosis in their study (23). Likewise, our group documented an activation of caspase-3 by HT in leukaemic CEM and REH cells and heat-induced cell death which was mainly due to caspase-dependent mechanisms (35, 36). An activation of caspase-3 in response to Mel treatment was recently reported by others (5, 14, 25). Kresse and colleagues found the cytotoxicity of Mel to be caspase-dependent (25). In contrast, our group found neither caspase-3 activation nor dependence of

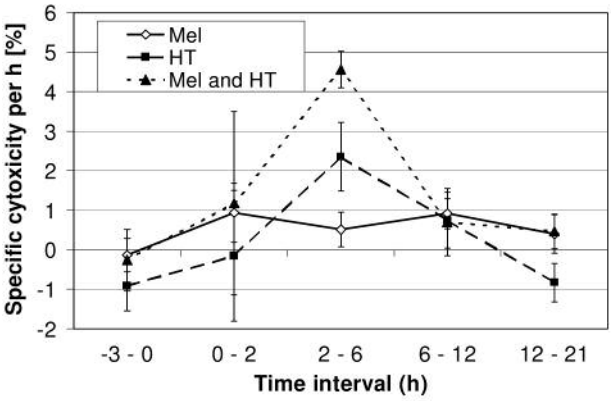

Figure 4. *Time-course of Mel- and HT-induced cytotoxicity. RD-ES cells were stimulated with Mel (10 μg/ml) and/or HT. AV/PI staining and flow cytometry were performed immediately after water bath incubation (0 h) and after an additional 2, 6, 12 or 21 h of culture under standard conditions. Results are expressed as the percentage of specific cytotoxicity, calculated on a per hour basis as described in the Materials and Methods section. Mean and SEM values of 7 independent experiments are shown.*

caspases for the cytotoxic effect of Mel in the rabdomyosarcoma cell line TE-671 (21). However, this cell line seemed to be resistant to Mel, as the drug was effective only at concentrations of 50 μg/ml and above, which cannot be achieved in clinical use. Cell death might have been due to necrosis in these cells triggered by very high drug concentrations.

In kinetic analyses, Mel induced a continuous level of cytotoxicity in RD-ES cells over 24 h. Heat alone mediated an early peak of cytotoxicity (at 2-6 h), whereas with the combination of Mel and HT after a high early peak (at 2-6 h), cytotoxicity decreased to the same level that was observed with Mel stimulation alone. It is therefore very likely that the early peak of cell death was mainly induced by the hyperthermic stimulus and the late elevation of cytotoxicity above baseline by Mel. In a previous study, our group demonstrated similar results in a human T-cell leukaemia line (CEM) with an early maximum of HT-mediated cell death directly after heat application and a later peak of cytotoxicity by cisplatin, both of which could be induced by a combined stimulus of heat and cisplatin in CEM cells (36). However, in the present study, the early cytotoxic peak of Mel and HT in combination was superadditive compared to the cytotoxicity of each stimulus given alone. This could be due to the recruitment of additional cytotoxic pathways by the combined stimulus in this time interval.

Using a commercial cDNA-Array, we investigated the gene expression in RD-ES cells 6 h after treatment with Mel and/or HT. Twenty out of 198 apoptosis-related genes featured in this gene array were identified to be regulated

Table I. *Effects of Mel and/or HT on apoptosis-related gene expression in RD-ES cells. Gene expression was analysed using the human apoptosis expression array, which contains the PCR products of 198 apoptosis-related genes. Six hours after stimulation with Mel (10 μg/ml) and/or HT, RNA was isolated from the RD-ES cells, reverse transcription was performed incorporating [α-^{32}P]-deoxycytidine-triphosphate, and the labelled cDNA was hybridised to the array membranes. Hybridisation signals were detected with a phosphor imager and normalised to the average signal of the housekeeping genes. Mean values of 3 independent experiments are shown.*

Gene family	Gene	Control	Mel	HT	Mel + HT
Apoptosis suppressor	XIAP	2.73	1.00	4.88	1.95
Apoptosis-related	eNOS	2.32	2.67	5.73	3.95
	TFAR15	1.55	2.28	4.08	0.77
Caspase-related	Caspase-3	2.08	4.77	3.72	4.60
	Caspase-8	1.57	2.13	4.32	2.38
Cell cycle regulator	P21	0.48	1.25	2.88	5.53
	PAK1B	11.27	4.50	13.88	4.78
	RB1	6.17	1.68	4.03	3.53
Cytokine	IGF-I R	2.90	0.18	4.35	1.27
	IL-1 R AcP	6.35	1.88	9.32	3.42
Signal transduction factor	Bcl-10	2.07	2.72	7.07	9.42
	DRAK2	1.65	1.82	2.57	4.40
	FAN	3.42	2.77	7.85	2.07
	par-4	6.18	4.18	13.67	4.27
	PTEN	6.30	5.12	14.67	5.90
TNF superfamily	4-1BBL	0.47	1.92	0.98	2.93
	Fas	7.27	5.88	18.40	7.37
	TRAIL-R1	1.73	2.52	3.70	1.87
	TRAIL-R2	12.42	18.18	34.93	23.75
	TRAIL-R4	6.97	17.28	14.10	15.93

increase ($x > 2.0$), which is significant ($p < 0.05$)
increase ($x > 2.0$), yet not significant
decrease ($x < 0.5$), which is significant ($p < 0.05$)
decrease ($x < 0.5$), yet not significant
slight changes of expression ($0.5 \leq x \leq 2.0$), which are significant ($p < 0.05$)

after stimulation with either Mel, heat or the combination of both. Mel suppressed the mRNA expression of *XIAP*, an important member of the inhibitors of apoptosis protein family, which act antiapoptotically through inactivation of active caspases (10, 11). Moreover, Mel mediated suppression of the retinoblastoma gene, whose gene product is reported to be antiapoptotic (6, 13), and two genes of the cytokine family (*IGF-1 R* and *IL-1R AcP*), which are also involved in pathways with antiapoptotic potency (2, 24, 26, 42, 46). The mRNA expression of the effector *caspase-3* and the tumour suppressor gene *p21* was enhanced after Mel-treatment. Although *TRAIL-R4*, an antiapoptotic gene (9, 32), was concomitantly induced, altogether the genetic regulation was driven towards apoptosis induction by Mel.

HT in principle induced genes involved in the extrinsic pathway of apoptosis. The mRNA expression of three death receptors of the tumour necrosis factor superfamily was up-regulated (*Fas, TRAIL-R1* and *TRAIL-R2*). Although the

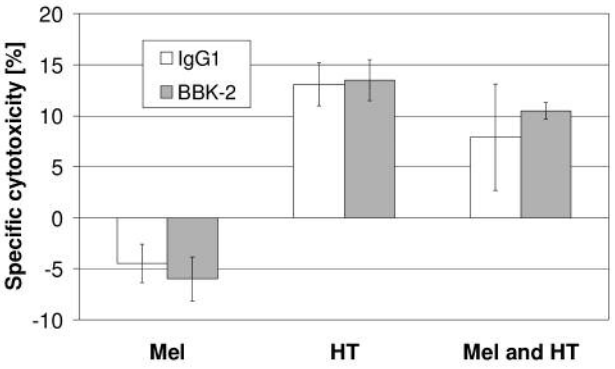

Figure 5. *Effect of inhibitory anti-CD137 antibody on Mel- and HT-induced cytotoxicity. RD-ES cells were preincubated for 1 h either with the nonstimulatory anti-CD137 antibody BBK-2 or with Ig-G1 as a control. The cells were then stimulated with Mel (10 μg/ml) and/or HT. Cytotoxicity was measured by AV/PI staining and flow cytometry 6 h after stimulation. Mean and SEM values show the specific cytotoxicity of 3 independent experiments.*

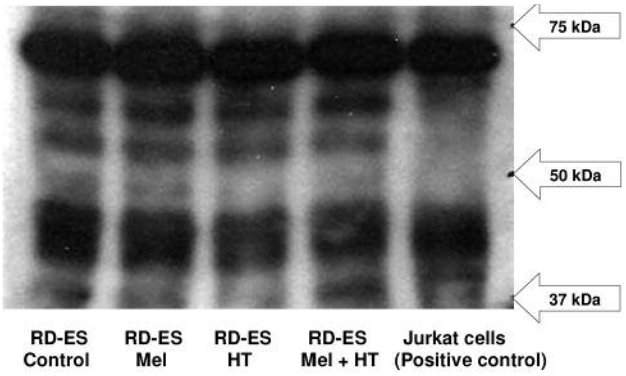

Figure 6. *DRAK2 protein expression in RD-ES cells. Cells were stimulated with Mel (10 μg/ml) and/or HT. Six hours after waterbath incubation, nuclear protein was extracted and protein levels were determined photometrically. SDS polyacrylamide electrophoresis and Western blot analysis with anti-DRAK2 antibody were performed. Jurkat cell lysate was used as a positive control. The blots represent one out of two separate experiments.*

decoy receptor *TRAIL-R4* was also induced by heat, the overall balance of pro-apoptotic and antiapoptotic receptors seemed to be regulated in favour of the death receptors. However, since the receptor profile does not always correlate with the response to TRAIL stimulation (9), the definitive effect of HT on the response to TRAIL cannot be predicted. HT also induced the transcription of *caspase-8*, which is the key initiator caspase of the receptor pathway (30). With *FAN* and *par-4* two other genes involved in the extrinsic apoptosis pathway were also induced; FAN mediates a communication with the synthesis pathway of pro-apoptotic ceramide (1) and par-4 regulates factors of the extrinsic as well as the intrinsic pathway in favour of apoptosis induction (12, 41).

The combination of Mel and HT led to a down-regulation of *PAK-1* which is a p21-activated kinase with antiapoptotic and mitogenic function (27). The expression of *bcl-10* was enhanced by HT with Mel as well as by heat given alone. Other genes that were significantly up-regulated by the combined stimulus were *CD137L* (4-1BBL) and *DRAK2*, for both of which a pro-apoptotic effect has been published (31, 33, 38, 49, 50). However, in our experiments, *CD137L* played no role in apoptosis induction in RD-ES cells, as the blockage of CD137-CD137L interaction did not affect the cell death rate. Moreover, at the protein level, CD137L was not detected on the surface of unstimulated or stimulated RD-ES cells. Salih and co-workers reported that CD137L can be released from the cell membranes in soluble form (48), which could be true for the RD-ES cells. The meaning of this release has not yet been elucidated.

DRAK2 protein expression was analysed by Western blotting of nuclear fractions of RD-ES cells. Thereby no clear difference in protein levels of DRAK2 with or without

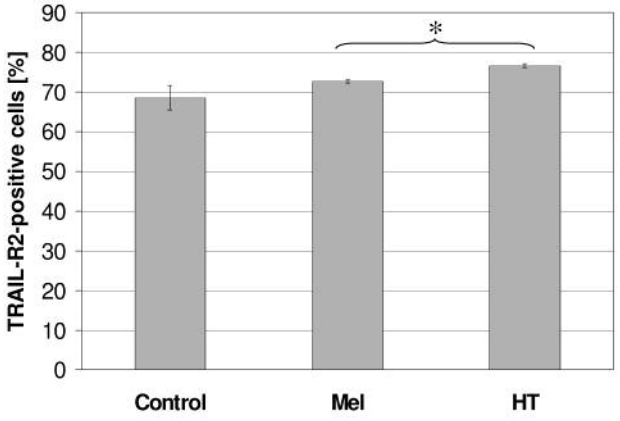

Figure 7. *Surface expression of TRAIL-R2 in unstimulated and stimulated RD-ES cells. Cell cultures were stimulated with Mel (10 μg/ml) or HT. After 6 h, surface expression of TRAIL-R2 was determined using a FITC-labelled anti-TRAIL-R2 antibody and flow cytometry. Mean and SEM values of 3 independent experiments are shown. Significant enhancement of TRAIL-R2 expression ($p<0.05$) is indicated by *.*

stimulation could be shown. DRAK2 was reported to be a nuclear protein (49). However, recently it has been found localized to the cytoplasm as well, although only the nuclear fraction seems to be relevant for apoptosis induction (28). The absence of an increase of protein expression despite enhanced mRNA levels in the cDNA array experiments could also be due to a latency period between transcription and translation of DRAK2 protein. Moreover, the low expression profile of DRAK2 and the fact that Western blotting as a semi-quantitative method cannot detect slight changes of protein expression might account for the lack of enhancement of DRAK2 protein levels.

The membrane expression of the pro-apoptotic TRAIL-receptor TRAIL-R2 was quantified by flow cytometry, since the particular mRNA level was remarkably high in unstimulated RD-ES cells and was further enhanced after heat treatment. Accordingly, TRAIL-R2 was highly expressed on the surface of unstimulated RD-ES cells with a percentage of 68.9±3.1% of cells being TRAIL-R2 positive. The heat-treated culture showed a slight enhancement of TRAIL-R2 expression, which was significantly higher than TRAIL-R2 expression after Mel stimulation, but showed only a trend to a difference compared to the control culture (p=0.054).

In conclusion, this study demonstrates for the first time that combination treatment with Mel and HT results in enhancement of antitumour activity against ET cells. Thermochemotherapy with Mel could therefore be a therapeutic option to intensify treatment for patients with HR-ET. However, further studies are needed to clarify the benefit and any possible side-effects of this therapy. Moreover, our results give insight into the complex genetic regulation in tumour cells in response to cytotoxic stimulation. Further studies based on the presented data could help to elucidate the divergent mechanisms involved in apoptosis induction and cell protection following cellular stress. In the present study, the different kinetic effects and gene regulation profiles after stimulation with Mel and HT suggest that these stimuli trigger different molecular pathways, which could explain the enhancement of cytotoxicity when both stimuli are applied in combination.

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References

1 Adam-Klages S, Adam D, Wiegmann K, Struve S, Kolanus W, Schneider-Mergener J and Krönke M: FAN, a novel WD-repeat protein, couples the p55 TNF-receptor to neutral sphingomyelinase. Cell *86*: 937-947, 1996.

2 Baserga R, Hongo A, Rubini M, Prisco M and Valentinis B: The IGF-I receptor in cell growth, transformation and apoptosis. Biochim Biophys Acta *1332*: F105-126, 1997.

3 Böhm P, Wirth CJ, Issels R, Riess H, Dressler W and Gokel JM: Operabilitat primar inoperabler Beckentumoren durch praoperative regionale Hyperthermie in Kombination mit systemischer Chemotherapie am Beispiel eines Ewing-Sarkoms. Chirurg *59*: 734-739, 1988.

4 Burdach S and Jürgens H: High-dose chemoradiotherapy (HDC) in the Ewing family of tumors (EFT). Crit Rev Oncol Hematol *41*: 169-189, 2002.

5 Chauhan D, Neri P, Velankar M, Podar K, Hideshima T, Fulciniti M, Tassone P, Raje N, Mitsiades C, Mitsiades N, Richardson P, Zawel L, Tran M, Munshi N and Anderson KC: Targeting mitochondrial factor Smac/DIABLO as therapy for multiple myeloma (MM). Blood *109*: 1220-1227, 2007.

6 Classon M and Harlow E: The retinoblastoma tumour suppressor in development and cancer. Nat Rev Cancer *2*: 910-917, 2002.

7 Debes A, Rommel F, Breise M, Willers R, Göbel U and Wessalowski R: *In vitro* test-system for chemo- and thermo-sensitivity: an analysis of survival fractions and cell-cycle distributions in human Ewing's sarcomas as a model for tumors in pediatric oncology. Klin Padiatr *214*: 223-229, 2002.

8 Debes A, Willers R, Göbel U and Wessalowski R: Role of heat treatment in childhood cancers: distinct resistance profiles of solid tumor cell lines towards combined thermochemotherapy. Pediatr Blood Cancer *45*: 663-669, 2005.

9 Degli-Esposti MA, Dougall WC, Smolak PJ, Waugh JY, Smith CA and Goodwin RG: The novel receptor TRAIL-R4 induces NF-kappaB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. Immunity *7*: 813-820, 1997.

10 Deveraux QL, Takahashi R, Salvesen GS and Reed JC: X-linked *IAP* is a direct inhibitor of cell-death proteases. Nature *388*: 300-304, 1997.

11 Eckelman BP, Salvesen GS and Scott FL: Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family. EMBO Rep *7*: 988-994, 2006.

12 El-Guendy N and Rangnekar VM: Apoptosis by Par-4 in cancer and neurodegenerative diseases. Exp Cell Res *283*: 51-66, 2003.

13 Fan G and Steer CJ: The role of retinoblastoma protein in apoptosis. Apoptosis *4*: 21-29, 1999.

14 Gokhalé P, Patel T, Morrison MJ and Vissers MCM: The effect of intracellular ascorbate on the susceptibility of HL60 and Jurkat cells to chemotherapy agents. Apoptosis *11*: 1737-1746, 2006.

15 Gorman AM, Heavey B, Creagh E, Cotter TG and Samali A: Antioxidant-mediated inhibition of the heat-shock response leads to apoptosis. FEBS Lett *445*: 98-102, 1999.

16 Hayes AJ, Neuhaus SJ, Clark MA and Thomas JM: Isolated limb perfusion with melphalan and tumor necrosis factor alpha for advanced melanoma and soft-tissue sarcoma. Ann Surg Oncol *14*: 230-238, 2007.

17 Hermisson M, Wagenknecht B, Wolburg H, Glaser T, Dichgans J and Weller M: Sensitization to CD95 ligand-induced apoptosis in human glioma cells by hyperthermia involves enhanced cytochrome *c* release. Oncogene *19*: 2338-2345, 2000.

18 Honess DJ and Bleehen NM: Thermochemotherapy with cis-platinum, CCNU, BCNU, chlorambucil and melphalan on murine marrow and two tumours: therapeutic gain for melphalan only. Br J Radiol *58*: 63-72, 1985.

19 Katschinski DM, Boos K, Schindler SG and Fandrey J: Pivotal role of reactive oxygen species as intracellular mediators of hyperthermia-induced apoptosis. J Biol Chem *275*: 21094-21098, 2000.

20 Klostergaard J, Leroux ME, Auzenne E, Khodadadian M, Spohn W, Wu JY and Donato NJ: Hyperthermia engages the intrinsic apoptotic pathway by enhancing upstream caspase activation to overcome apoptotic resistance in MCF-7 breast adenocarcinoma cells. J Cell Biochem *98*: 356-369, 2006.

21 Klüttermann K, Banning U, Kachel M, Krause C, Körholz D and Mauz-Körholz C: TRAIL-induced cytotoxicity in a melphalan-resistant rhabdomyosarcoma cell line *via* activation of caspase-2. Anticancer Res *26*: 351-356, 2006.

22 Kobayashi D, Sasaki M and Watanabe N: Caspase-3 activation downstream from reactive oxygen species in heat-induced apoptosis of pancreatic carcinoma cells carrying a mutant *p53* gene. Pancreas *22*: 255-260, 2001.

23 Kobayashi D, Watanabe N, Yamauchi N, Okamoto T, Tsuji N, Sasaki H, Sato T and Niitsu Y: Heat-induced apoptosis *via* caspase-3 activation in tumour cells carrying mutant *p53*. Int J Hyperthermia *16*: 471-480, 2000.
24 Kothny-Wilkes G, Kulms D, Luger TA, Kubin M and Schwarz T: Interleukin-1 protects transformed keratinocytes from tumor necrosis factor-related apoptosis-inducing ligand- and CD95-induced apoptosis but not from ultraviolet radiation-induced apoptosis. J Biol Chem *274*: 28916-28921, 1999.
25 Kresse M, Latta M, Künstle G, Riehle H, van Rooijen N, Hentze H, Tiegs G, Biburger M, Lucas R and Wendel A: Kupffer cell-expressed membrane-bound TNF mediates melphalan hepatotoxicity *via* activation of both TNF receptors. J Immunol *175*: 4076-4083, 2005.
26 Kühn K, Hashimoto S and Lotz M: IL-1 beta protects human chondrocytes from CD95-induced apoptosis. J Immunol *164*: 2233-2239, 2000.
27 Kumar R, Gururaj AE and Barnes CJ: p21-Activated kinases in cancer. Nat Rev Cancer *6*: 459-471, 2006.
28 Kuwahara H, Nakamura N and Kanazawa H: Nuclear localization of the serine/threonine kinase DRAK2 is involved in UV-induced apoptosis. Biol Pharm Bull *29*: 225-233, 2006.
29 Laurence V, Pierga J, Barthier S, Babinet A, Alapetite C, Palangié T, de Pinieux G, Anract P and Pouillart P: Long-term follow up of high-dose chemotherapy with autologous stem cell rescue in adults with Ewing tumor. Am J Clin Oncol *28*: 301-309, 2005.
30 Lavrik I, Golks A and Krammer PH: Death receptor signaling. J Cell Sci *118*: 265-267, 2005.
31 Mao J, Qiao X, Luo H and Wu J: Transgenic *drak2* overexpression in mice leads to increased T-cell apoptosis and compromised memory T-cell development. J Biol Chem *281*: 12587-12595, 2006.
32 Marsters SA, Sheridan JP, Pitti RM, Huang A, Skubatch M, Baldwin D, Yuan J, Gurney A, Goddard AD, Godowski P and Ashkenazi A: A novel receptor for Apo2L/TRAIL contains a truncated death domain. Curr Biol *7*: 1003-1006, 1997.
33 Matsumoto M, Miyake Y, Nagita M, Inoue H, Shitakubo D, Takemoto K, Ohtsuka C, Murakami H, Nakamura N and Kanazawa H: A serine/threonine kinase which causes apoptosis-like cell death interacts with a calcineurin B-like protein capable of binding Na(+)/H(+) exchanger. J Biochem (Tokyo) *130*: 217-225, 2001.
34 Mauz-Körholz C, Dietzsch S, Banning U, Tröbs RB and Körholz D: Mechanisms of hyperthermia- and 4-hydroperoxy-ifosfamide-induced cytotoxicity in T-cell leukemia. Anticancer Res *22*: 4243-4247, 2002.
35 Mauz-Körholz C, Dietzsch S, Banning U, Tröbs R and Körholz D: Heat- and 4-hydroperoxy-ifosfamide-induced apoptosis in B-cell precursor leukaemias. Int J Hyperthermia *19*: 444-460, 2003.
36 Mauz-Körholz C, Dietzsch S, Schippel P, Banning U and Körholz D: Molecular mechanisms of hyperthermia- and cisplatin-induced cytotoxicity in T-cell leukemia. Anticancer Res *23*: 2643-2647, 2003.
37 Meyer T and Göhl J: Regionale Chemotherapie-Extremitäten-perfusion. Kongressbd Dtsch Ges Chir Kongr *118*: 200-204, 2001.
38 Michel J, Pauly S, Langstein J, Krammer PH and Schwarz H: CD137-induced apoptosis is independent of CD95. Immunology *98*: 42-46, 1999.
39 Mohamed F, Marchettini P, Stuart OA, Urano M and Sugarbaker PH: Thermal enhancement of new chemotherapeutic agents at moderate hyperthermia. Ann Surg Oncol *10*: 463-468, 2003.
40 Mohamed F, Stuart OA, Glehen O, Urano M and Sugarbaker PH: Optimizing the factors which modify thermal enhancement of melphalan in a spontaneous murine tumor. Cancer Chemother Pharmacol *58*: 719-724, 2006.
41 Mundle SD: Par-4: a common facilitator/enhancer of extrinsic and intrinsic pathways of apoptosis. Leuk Res *30*: 515-517, 2006.
42 O'Connor R: Regulation of IGF-I receptor signaling in tumor cells. Horm Metab Res *35*: 771-777, 2003.
43 Paulussen M, Fröhlich B and Jürgens H: Ewing tumour: incidence, prognosis and treatment options. Paediatr Drugs *3*: 899-913, 2001.
44 Pinguet F, Martel P, Fabbro M, Petit I, Canal P, Culine S, Astre C and Bressolle F: Pharmacokinetics of high-dose intravenous melphalan in patients undergoing peripheral blood hematopoietic progenitor-cell transplantation. Anticancer Res *17*: 605-611, 1997.
45 Pinkerton CR, Bataillard A, Guillo S, Oberlin O, Fervers B and Philip T: Treatment strategies for metastatic Ewing's sarcoma. Eur J Cancer *37*: 1338-1344, 2001.
46 Reddy SAG, Lin Y, Huang HJ, Samanta AK and Liao WS: The IL-1 receptor accessory protein is essential for PI 3-kinase recruitment and activation. Biochem Biophys Res Commun *316*: 1022-1028, 2004.
47 Rothbarth J, Tollenaar RAEM, Schellens JHM, Nortier JWR, Kool LJS, Kuppen PJK, Mulder GJ and van de Velde CJH: Isolated hepatic perfusion for the treatment of colorectal metastases confined to the liver: recent trends and perspectives. Eur J Cancer *40*: 1812-1824, 2004.
48 Salih HR, Schmetzer HM, Burke C, Starling GC, Dunn R, Pelka-Fleischer R, Nuessler V and Kiener PA: Soluble CD137 (4-1BB) ligand is released following leukocyte activation and is found in sera of patients with hematological malignancies. J Immunol *167*: 4059-4066, 2001.
49 Sanjo H, Kawai T and Akira S: DRAKs, novel serine/threonine kinases related to death-associated protein kinase that trigger apoptosis. J Biol Chem *273*: 29066-29071, 1998.
50 Schwarz H, Blanco FJ, von Kempis J, Valbracht J and Lotz M: ILA, a member of the human nerve growth factor/tumor necrosis factor receptor family, regulates T-lymphocyte proliferation and survival. Blood *87*: 2839-2845, 1996.
51 Takemoto M, Kuroda M, Urano M, Nishimura Y, Kawasaki S, Kato H, Okumura Y, Akaki S, Kanazawa S, Asaumi J, Joja I and Hiraki Y: The effect of various chemotherapeutic agents given with mild hyperthermia on different types of tumours. Int J Hyperthermia *19*: 193-203, 2003.
52 Urano M, Kuroda M and Nishimura Y: For the clinical application of thermochemotherapy given at mild temperatures. Int J Hyperthermia *15*: 79-107, 1999.
53 Urano M and Ling CC: Thermal enhancement of melphalan and oxaliplatin cytotoxicity *in vitro*. Int J Hyperthermia *18*: 307-315, 2002.
54 Vertrees RA, Das GC, Coscio AM, Xie J, Zwischenberger JB and Boor PJ: A mechanism of hyperthermia-induced apoptosis in ras-transformed lung cells. Mol Carcinog *44*: 111-121, 2005.

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