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Anticancer Efficacies of Doxorubicin, Verapamil and Quercetin on FM3A Cells under Hyperthermic Temperature

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Abstract Hyperthermia (HT) in combination with anticancer drugs (ACDs) had proven to more efficacious in various cancers, although efficacies vary according to chemotherapeutic compounds and cancer types. Presently there are few data that compares anticancer efficacies among ACDs under hyperthermic conditions. Therefore, we selected three commonly used ACDs (quercetin, verapamil and doxorubicin) and compared their antitumor effects when each was treated with 43°C HT exposure. Firstly, FM3A, a murine breast cancer cell line, was treated with each ACD for 1 h followed by 43°C exposure for additional 1 h, and examined the effects of: 1) each drug, 2) 43°C HT exposure, and 3) the combination of each drug and 43°C HT exposure for 1, 6 and 24 h. The determined overall effects on FM3A cells were arrested cell proliferation, clonogenic efficiency and apoptosis. Pre-treatment of FM3A cells to each ACD followed by 43°C HT exposure produced greater antitumor effects including suppressed cell proliferation, reduced clonogenic efficiency and increased apoptotic cell death, compared to ACD treatment or HT exposure alone. Apoptotic cell death occurred in a time-dependent manner. Among the ACDs, antitumor efficacies varied in the order of doxorubicin > verapamil > quercetin. It was concluded that heat exposure during ACD treatment of caner cells may be an important factor to get a better antitumor benefit, even though this benefit may differ from one drug to another.

Keywords: hyperthermia, FM3A cells, anticancer drugs, apoptosis, cancer, cytotoxicity

INTRODUCTION

Hyperthermia (HT) in combination with anticancer drugs (ACDs) is thought to offer a promising alternative therapy for cancers [1]. Several studies have given basis for these thoughts by recently shown that the combination of HT and ACDs is more efficacious in the treatment of various cancers [2-4]. Although it has been known for several decades that tumor cells are more heat-sensitive than normal cells [5-7], the reasons for enhancement of anticancer activity by heat are still poorly understood [8]. When used simultaneously with ACDs, it is shown that heat increases intracellular drug penetration and enhances the cytotoxic effects of the ACDs [9]. Although it is known that potentiation of cancer drug cytotoxicity by heat differs from compound to compound [9], there are few data that compare the cytotoxicity of ACDs during cancer cell exposure under hyperthermic conditions. We therefore selected three antitumor compounds used commonly, doxorubicin (DOX), verapamil (VEP) and quercetin (QCT), and conducted a comparative evaluation of their antitumor activities when FM3A cells were exposed simultaneously at 43°C.

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MATERIALS AND METHODS

Reagents

RPMI 1640 and foetal bovine serum (FBS) were obtained from Life Technologies (Grand Island, NY, USA). VEP, QCT, DOX, propidium iodide (PI) and ribonuclease A (RNase A) were from the Sigma Chemical Co. (St. Louis, MO, USA).

In vitro Cell Culture and Hyperthermic Treatment

FM3A mouse mammary cancer cell line, which was obtained from Riken Cell Bank (Tsukuba, Japan), was maintained in RPMI 1640 media supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C, and in a humidified atmosphere of 5% CO_2 and 95% air. For the experiments, FM3A cells (~2 × 10^6 cells/mL) were seeded in 50 mL screw topped polystyrene

flasks (Falcon; Franklin Lakes, NJ, USA) in RPMI 1640 medium and were either incubated at 37°C (control) or RPMI 1640 medium containing 20 μM of ACD solutions at 37 and 43°C (tests). A 20 μM solutions of the drugs were prepared from stock solutions using RPMI 1640 media. Before cell inoculations, all media were preheated to the final temperature of incubation. For hyperthermic treatment, cells were initially incubated in ACD solutions at 37°C for 1 h prior to the 43°C heat exposure.

Determination of Percentage of Apoptotic Cells and Cell Cycle Phases

Flow cytometric analysis was used for the assessment of the percentage of apoptotic (hypodiploid) cells among diploid cells as well as cell cycle phases. After treating ACDs followed with HT exposures, 1 mL of medium containing ~2 × 10^6 cells was transferred to a 1.5 mL sterile microcentrifuge tubes with triplicates for each of the test conditions. After centrifugation of microcentrifuge tubes at 1000 rpm for 5 min, cell pellets were washed twice with ice cold phosphate buffered saline (PBS) solution, and then were resuspended in 400 μL of ice cold PBS. The cells were further diluted with 400 μL of 70% ethanol (by drop wise addition while vortexing), and the resulting suspensions were allowed to fix for 24 h at 4°C. After fixing, the cells were spun down at 2000 rpm, and washed twice with ice cold PBS. The pellets were resuspended in 400 μL of 0.1% (w/v) sodium citrate containing 1 mg/mL RNase A (Sigma; St. Louis, MO, USA), and then were incubated at 37°C for 20 min. Final cell suspensions were stored at -20°C until flow cytometric analysis. Immediately before flow cytometry, the cells were spun down at 1000 rpm for 5 min, and the pellets were resuspended in 400 μL of 0.1% (w/v) sodium citrate that contained 100 μg/mL PI. The cells were incubated in the dark at 21°C for 15 min, and then were examined with Becton Dickinson FACScan™ (BD Biosciences; San Jose, CA, USA) using a excitation wavelength of 488 nm, gating out doublets and clumps using pulse emitting and collecting fluorescence (emission wavelength) of 585 ± 21 nm. Cells with a DNA content that was less than the diploid cells in the G_0/G_1 phase were considered apoptotic. DNA histograms and cell cycle phases were analyzed using CellFIT software (BD Biosciences; San Jose, CA, USA). The median values were estimated by two different methods: RFIT and SFIT.

Cell Proliferation and Colony Forming Assays

Cell proliferation assay was performed by inoculating 100,000 cells into a 10 mL culture flask (10,000 cells/mL) from each sample after ACD treatment and heat exposure. The flasks were then incubated for 5 days. Changes in cell numbers of each sample were observed every 24 h using the new improved Neubauer Hemocytometer. The percentage of cell proliferation inhibition is calculated by the following equation:

$$\text{Inhibition of cell proliferation (\%)} = \frac{100 \times (\text{Number of cells in treated samples})}{(\text{Number of cells in control samples})}$$

To estimate clonogenic efficiency, 1000 cells from each sample were seeded in 3 mL of RPMI 1640 media containing 0.33% agar and overlayered with 4 mL of RPMI 1640 medium containing 0.5% agar in a 60 mm tissue culture dishes (Iwaki Glass; Yokohama, Japan). Then, the culture dishes were incubated under regular culture conditions for 9 days, and then stained with the Giemsa stain (Sigma; St. Louis, MO, USA). The aggregate that consisted of 20 or more cells was counted as a colony. The results are expressed as the mean number of colonies from 3 triplet plates. The suppression of colony formation was calculated with the following equation:

$$\text{Suppression of colony formation (\%)} = \frac{100 \times (\text{Number of colonies in treated samples})}{(\text{Number of colonies in control samples})}$$

Evaluation of Combined Effects of HT Exposure and ACD Treatment

The combined effects of HT exposure was evaluated using Valeriote's method [10] with the following standards,
[C] < [E]: Synergistic,
[C] = [E]: Synergistic,
[E] < [C] < [H] or [E] < [C] < [D]: Sub-synergistic,
[D] < [C] < [H]: Interference,
where [H], [D], and [C] represent variability in the cases of HT exposure alone, ACD treatment alone and the combination of hyperthermia and drug, respectively. [E] (=[H]×[D]/100) is the expected variability in the case of the combination.

Statistical Analysis

Differences in the degree of cell proliferation and colony formation between the control and test groups were analyzed by ANOVA using the statistical package STATVIEW (Abacus Concepts Inc., Berkerly, CA, USA).

RESULTS

Effects of ACD Treatment with 43°C HT Exposure on Apoptosis

A flow cytometric method for simultaneous detection of apoptotic cell and cell cycle was applied on the FM3A cell line. Four ACDs currently used in the treatment of cancers were studied *in vitro*: DOX, VEP, and QCT. The effects of 20 μM of DOX, VEP, QCT, and 43°C HT exposure, as well as the combination of each ACD with

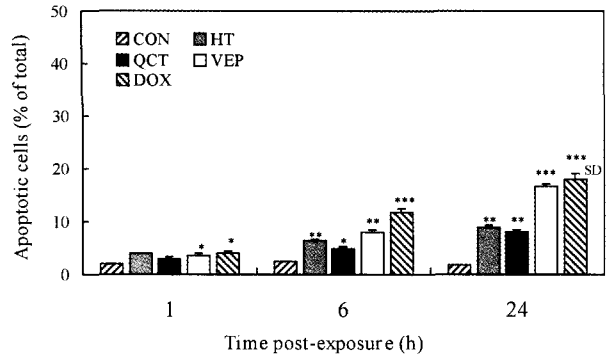

Fig. 1. Apoptosis histograms showing responses of FM3A cells to quercetin (QCT), verapamil (VEP), and doxorubicin (DOX) treatment for 1, 6, and 24 h without hyperthermia (HT) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

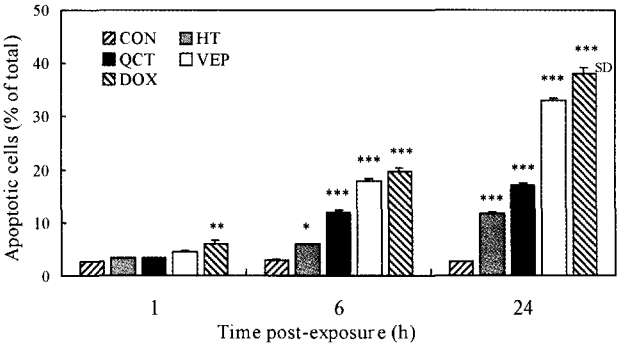

Fig. 2. Apoptosis histograms showing responses of FM3A cells to hyperthermic (HT) exposure alone or in combination with quercetin (QCT), verapamil (VEP), and doxorubicin (DOX) treatment for 1, 6, and 24 h (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

43°C HT exposure on apoptosis after 1, 6, and 24 h are presented in Figs. 1 and 2. Our results show that the strength of apoptotic effects of the drugs increases in the order of DOX > VEP > QCT. There was no significant apoptotic effect in case of 43°C HT exposure alone. In case of ACD treatment, no significant changes were observed after 1 h, but the percentage of apoptotic cells increased according as time passed. Moreover, when the FM3A cells were incubated in the ACD solution prior to 43°C HT exposure, the percentage of PI-positive cells increased as much as 2 times of ACD treatment alone (Figs. 1 and 2). After 24 h, significant nuclear fragmentation was observed (data not shown), which coincided with the induction of apoptosis. It could be concluded that 1 h of treatment was sufficient to induce apoptosis by DOX ($P < 0.05$) and VEP ($P < 0.05$).

Effects of ACD Treatment with 43°C HT Exposure on Cell Growth and Clonogenic Efficiency

The growth inhibitory effects of ACD treatment alone

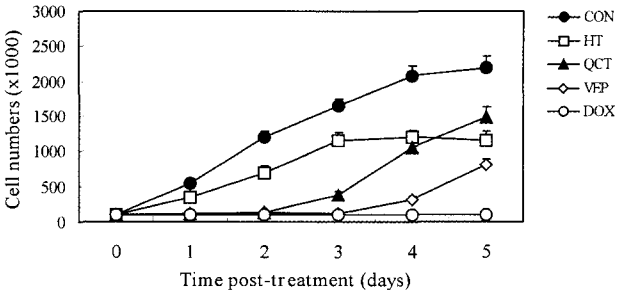

Fig. 3. Cell growth curves of FM3A cells were treated with doxorubicin (DOX), verapamil (VEP), and quercetin (QCT) without pre-exposure at 43°C and were cultured at 37°C for 5 days. CON: control, HT: 43°C exposure only without anticancer drugs.

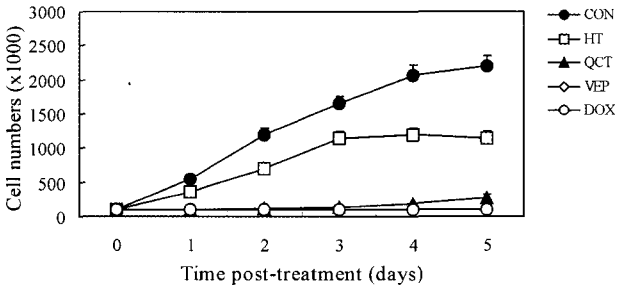

Fig. 4. Cell growth curves of FM3A cells were treated with doxorubicin (DOX), verapamil (VEP), and quercetin (QCT) for 1 h and then exposed at 43°C for 1 h, and then, were cultured at 37°C for 5 days. CON: control, HT: 43°C exposure only without anticancer drugs.

and its combination with 43°C HT exposure are shown in Figs. 3 and 4. FM3A cells were found to be 132, 1.8, and 1.3 times more sensitive to DOX and VEP as opposed to QCT (Fig. 3). In general the cytostatic and clonogenic effects of the drugs are as follows: (a) DOX exhibited a markable antiproliferative effect during the 5 days of exposure, whereas VEP and QCT suppressed the increase of cell numbers for the first 72 h, which was followed by a sharp increase. For HT exposure, there was a steady increase in cell numbers for the first 72 h, and then the cell proliferation reached plateau across the next 48 h. QCT treatment and HT exposure showed fairly similar maximum cell proliferation at day 5. From this, it was found that DOX appear to successfully suppress cell proliferation without HT exposure, VEP and QCT applied individually were relatively ineffective in inhibiting the *in vitro* growth of FM3A cells, giving growth inhibitions varying between 46 and 78%.

When DOX, VEP, and QCT treatment were followed by 43°C HT exposure for 1 h, almost 100% inhibition of cell growth was achieved (Fig. 4). The sensitivity of FM3A cell growth on 43°C HT exposure, ACD treatment alone, and their combination correlated well with their respective clonogenic efficiencies, in which pre-treatment of

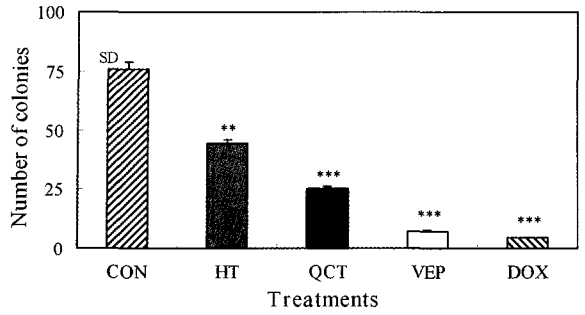

Fig. 5. Clonogenic assay of FM3A cells were treated with doxorubicin (DOX), verapamil (VEP), and quercetin (QCT) for 1 h followed by exposure at 43°C for 1 h, and then, were cultured in 35 mm dish at 37°C for 10 days. CON: control, HT: 43°C exposure only without anticancer drugs (**, $P < 0.01$; ***, $P < 0.001$).

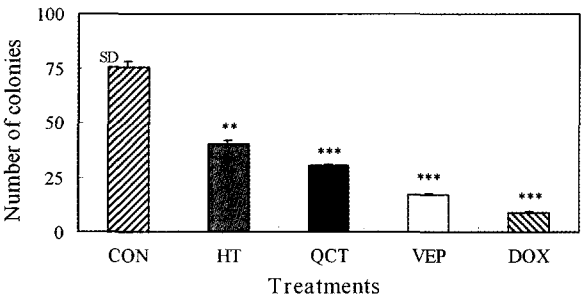

Fig. 6. Clonogenic assay of FM3A cells were treated with doxorubicin (DOX), verapamil (VEP), and quercetin (QCT) for 1 h without heat exposure at 43°C and were cultured in a 35 mm dish at 37°C for 10 days. CON: control, HT: 43°C exposure only without anticancer drugs (**, $P < 0.01$; ***, $P < 0.001$).

each ACD followed by 43°C HT exposure exerted a synergistic suppression of colony formation (Figs. 5 and 6).

DISCUSSION

Apoptosis, often synonymously used with the term 'programmed cell death', is an active, genetically controlled process that removes unwanted or damaged cells. Alterations in the expression of genes affecting cell cycle progression occur in all human cancers [11]. These may occur either by overexpression of genes such as cyclin D1, mutation of regulatory genes such as p16, or abrogation of checkpoints following DNA damage as in the cases of mutation or deletion of the p53 gene [12]. Perturbation of the normal functions of these genes has a profound effect on cellular proliferation, differentiation and apoptosis. There is increasing evidence that the increased level of cell survival in cancer (*i.e.* associated with inhibition of apoptosis) may reduce the cellular response to treatment with chemotherapeutic agents. The results from this study reveal that DOX appear to successfully suppress cell proliferation, whereas VEP and QCT did not without HT exposure. In case of ACD treatment followed by 43°C HT exposure for 1 h, greater than 85% inhibitions in cell growth was observed (Figs. 3 and 4). With respect to molar concentrations used, weak antiproliferative effects of VEP and QCT against FM3A cells were evident, but DOX showed a more impressive effect. Treatments with VEP and QCT produced less than 65% cell death whereas DOX produced greater than 80% decrease of cell numbers, which confirms the superior cytotoxic effects of DOX. The contributory effects of HT alone may provide some kind of synergisms to the individual ACDs.

Although HT exposure itself is cytotoxic, it also increases the cytotoxicity of ACDs, though the magnitude of this contribution varies depending on the drug or cell type. In addition to the data presented in this study, this hyperthermic property has been widely reported in other literatures. For instance HT (42°C) exposure alone clearly increased the cytotoxicity of adriamycin in AuxB1 cells and in prostate cancer [13], but caused only a slight increase of toxicity in Chinese hamster ovary cells at 42 and 43°C [14]. Equally, HT exposure enhanced the cytotoxic effects of DOX much more on murine leukaemia cell lines than other cells [15]. In terms of drug terms, VEP individually did not show cytotoxicity to either Ehrlich ascites carcinoma cells, or Sarcoma 180 tumor cells. However, a combination of bouvardin and VEP at an elevated temperature resulted in a greater amount of cell death in the Ehrlich ascites carcinoma cells, but the natural resistance of Sarcoma 180 tumor cells to bouvardin at HT condition was circumvented by the combination with VEP. The combination of bouvardin and VEP treatment with HT exposure resulted in a greater amount of cell death, compared to separate treatments of single agent [16].

Wide differences in cytotoxic properties have also been observed among the flavones. Cancer cells produce large amounts of lactate *via* aerobic glycolysis and the resulting acidic pH has been shown to selectively enhance the cytotoxic effects under HT condition [17]. QCT inhibits lactate transport and lowers intracellular pH, and thus, it enhances the cytotoxic effects by HT. However, QCT was not cytotoxic up to 4 h at 37°C (0.1 mM). When HeLa cells were exposed to QCT at 41 and 42°C, significant potentiation was dependent on the drug concentration, pH of the culture medium, temperature and duration of treatment [18]. But rutin, a structurally related bioflavonoid of QCT that lacks the property of lactate transport inhibition, showed no HT potentiation [19]. Illustrating further, both DND-1A human malignant melanoma and DND-39A Burkitt's lymphoma cell lines differed in sensitivity when exposed to heat [20]. With the malignant melanoma cells, HT potentiated the cytotoxic effects of the anthracycline antibiotics DOX, daunorubicin, mitoxantrone and quelamycin, but did not enhance the cytotoxic effects of aclacinomycin [20].

Drug resistance, which so often accompanies with tumor progression, has shown to be related to changes in membrane properties, especially to decreased drug accumulation in the tumor cell [21]. The increase of intra-

cellular drug accumulation by HT is possibly due to an increase in the cell membrane potential and the inhibition of the drug efflux, to reverse drug resistance [22]. Significantly less amount of P-glycoprotein and metallothionein expression were reported in cells preheated at 44°C than in control cells or cells that were preheated at 41°C ($P < 0.01$) [23]. This reduction in P-glycoprotein and metallothionein expression by heat may contribute in inhibiting drug efflux and thus increase intracellular drug level at elevated temperatures. Thus, HT exposure may give damage the drug-exclusion mechanism and increase the effectiveness of the drug action [13]. Drug accumulation also varies in different cells. For instance, HT significantly increased the intracellular amounts of the antitubulin agents, vinblastine and docetaxel, in the MESSA cell and, to a much lesser extent, in K562 cells. However, multi-drug resistant cells retained a profound drug accumulation defect at 43°C [9]. The cytotoxic role of the temperature was further illustrated by the fact that exposure of cells to the anthracycline compounds at 0°C resulted in almost a complete disappearance of cell killing effects [24].

It is also important to realize that duration time of HT exposure is very critical. Exposing cells first to HT before the drug treatment resulted in thermotolerance. Thermotolerant cells showed a significantly better survival rate when ACD treatment was combined with HT exposure. Probably, the enhanced cell survival is correlated with the accumulation of inducible HSP70 [13].

Therefore, it is apparent that stronger inhibitory and cytotoxic activities would obtained by using doses higher than 20 μM, because ACD exerts its cytotoxic effect in a concentration-dependent manner in most cells [25,26]. There was a significant difference in anticancer effects when FM3A cancer cells were treated with DOX, VEP, and QCT, and the cytotoxic effects were increased in the order of DOX > VEP > QCT. The most likely reason for the difference in cytotoxicity despite of equimolar concentrations may possibly be related to the cytotoxic mechanisms of the drugs. However, it is also known that, irrespectively the primary action site of a drug, cell death by most pharmacological agents is mediated by the activation of the signal transduction pathway for apoptosis [26-30]. Regardless the action of the drug on signal pathways for apoptosis, the severity of apoptosis was proportional to the length of time for HT post-exposure (Fig. 2). On the other hand, the dose necessary to induce cell death depends on the sensitivity of the cells to the drug. For instance, Kang and Liang [26] observed a 52.7% of cell death with 20 μM QCT after *in vitro* incubation of human leukemia (HL-60) cells for 96 h. However, in another study, 50% of cell death on human breast cancer was achieved with the use of doses higher than 500 μM [30].

CONCLUSION

HT exposure appears to increase sensitivity in cancer treatment by using chemotherapeutic drugs. It is crucial to conduct further studies that aid the understanding of the interactions between drug mechanisms of action and the role of HT exposure in order to exploit potential benefits.

REFERENCES

[1] Kobayashi, T. (1993) Hyperthermia for brain tumors. *Jpn. J. Hyperther. Oncol.* 9: 245-249.

[2] Fujita. M., M. Nagai, M. Murata, K. Kawakami, S. Irino, and J. Takahara (1997) Synergistic cytotoxic effect of quercetin and heat treatment in a lymphoid cell line (OZ) with low HSP70 expression. *Leuk. Res.* 21: 139-145.

[3] Yanase, M., M. Shinkai, H. Honda, T. Wakabayashi, J. Yoshida, and T. Kobayashi (1998) Intracellular hyperthermia for cancer using magnetite catiotic liposomes: An *in vivo* study. *Jpn. J. Cancer Res.* 89: 463-469.

[4] Yamamoto, M., M. Kuroda, O. Honda, E. Ono, J. I. Asaumi, K. Shibuya, S. Kawasaki, I. Joja, M. Takemoto, S. Kanazawa, and Y. Hiraki (1999) Cepharanthin enhances thermosensivity without a resultant reduction in thermotolerance of a murine mammary carcinoma. *Int. J. Oncol.* 15: 95-99.

[5] Overgaard, K. and J. Overgaard (1972) Investigations on the possibility of a thermic tumor therapy. I. Short-wave treatment of transplanted isologous mouse mammary carcinoma. *Eur. J. Cancer* 8: 65-78.

[6] Overgaard, J. (1977) Effect of hyperthermia on malignant cells *in vivo*: A review and a hypothesis. *Cancer* 39: 2637-2646.

[7] Dessureault, S., P. Brown, T. Ghose, O. P. Kamra, and P. Belitsky (1988) Sensitivity of normal and neoplastic human renal tubular epithelium to hyperthermia. *Cancer Lett.* 43: 1-8.

[8] Cajone, F., S. Debiasi, C. Parker, M. S. Lakshmi, and G. V. Sherbet (1994) Metastasis-associated mts1 gene expression is down-regulated by heat shock in variant cell lines of the B16 murine melanoma. *Melanoma. Res.* 4: 143-150.

[9] Dumontet, C., F. Bodin, and Y. Michal (1998) Potential interactions between antitubulin agents and temperature: implications for modulation of multidrug resistance. *Clin. Cancer Res.* 4: 1563-1566.

[10] Valeriote, F. and H. Lin (1975) Synergistic interaction of anticancer agents: a cellular perspective. *Cancer Chemother. Rep.* 59: 895-900.

[11] Kim, E. J. and T. H. Park (2003) Anti-apoptosis engineering. *Biotechnol. Bioprocess Eng.* 8: 76-82.

[12] Hochhauser, D. (1997) Modulation of chemosensitivity through altered expression of cell cycle regulatory genes in cancer. *Anticancer Drugs* 8: 903-910.

[13] Roigas, J., E. S. Wallen, S. A. Loening, and P. L. Moseley (1998) Effects of combined treatment of chemotherapeutics and hyperthermia on survival and the regulation of heat shock proteins in Dunning R3327 prostate carcinoma cells. *Prostate* 34: 195-202.

[14] Averill, D. A. and C. Su (1999) Sensitization to the cytotoxicity of adriamycin by verapamil and heat in multidrug-resistant Chinese hamster ovary cells. *Radiat. Res.* 151:

694-702.
[15] Toffoli, G., C. Bevilacqua, A. Franceschin, and M. Boiocchi (1989) Effect of hyperthermia on intracellular drug accumulation and chemosensitivity in drug-sensitive and drug- resistant P388 leukaemia cell lines. *Int. J. Hyperther.* 5: 163-172.
[16] Chitins, M. P. and M. K. Adwankar (1986) Enhancement of the in vitro cytotoxicity of bouvardin by verapamil alone and combined with hyperthermia in Sarcoma 180 and Ehrlich ascites carcinoma cells. *J. Cancer Res. Clin. Oncol.* 112: 131-134.
[17] Burdon, R. H., S. M. Kerr, C. M. Cutmore, J. Munro, and V. Gill (1984) Hyperthermia, Na^+K^+ ATPase and lactic acid production in some human tumour cells. *Br. J. Cancer* 49: 437-445.
[18] Kim, J. H., S. H. Kim, A. A. Alfieri, and C. W. Young (1984) Quercetin, an inhibitor of lactate transport and a hyperthermic sensitizer of HeLa cells. *Cancer Res.* 44: 102-106.
[19] Belt, J. A., J. A. Thomas, R. N. Buchsbaum, and E. Racker (1979) Inhibition of lactate transport and glycolysis in Ehrlich ascites tumor cells by bioflavonoids. *Biochemistry* 18: 3506-3511.
[20] Ohnoshi, T., T. Ohnuma, J. T. Beranek, and J. F. Holland (1985) Combined cytotoxicity effect of hyperthermia and anthracycline antibiotics on human tumor cells. *J. Natl. Cancer Inst.* 74: 275-281.
[21] Leibovici, J., G. Klorin, M. Huszar, S. Hoenig, O. Klein, M. Michowitz, and A. Pinchassov (1990) Sensitivity to thermochemotherapy of AKR lymphoma and B16 melanoma variants of malignancy. *Clin. Exp. Metastasis* 8: 33-46.
[22] Asaumi, J., S. Kawasaki, K. Nishikawa, M. Kuroda, and Y. Hiraki (1995) Effects of hyperthermia and cepharanthin on adriamycin accumulation with changes in extracellular pH. *Int. J. Hyperther.* 11: 27-35.
[23] Moriyama-Gonda, N., M. Igawa, H. Shiina, and Y. Wada (1998) Heat-induced membrane damage combined with adriamycin on prostate carcinoma PC-3 cells: correlation of cytotoxicity, permeability and P-glycoprotein or metallothionein expression. *Br. J. Urol.* 82: 552- 559.
[24] Lane, P., P. Vichi, D. L. Bain, and T. R. Tritton (1987) Temperature dependence studies of adriamycin uptake and cytotoxicity. *Cancer Res.* 47: 4038-4042.
[25] Urano, M., J. Begley, and R. Reynolds (1994) Interaction between adriamycin cytotoxicity and hyperthermia: growth-phase-dependent thermal sensitization. *Int. J. Hyperther.* 10: 817-826.
[26] Kang, T. B. and N. C. Liang (1997) Studies on the inhibitory effects of quercetin on the growth of HL-60 leukemia cells. *Biochem. Pharmacol.* 54: 1013-1018.
[27] Barry, M. A., C. A. Behnke, and A. Eastman (1990) Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia. *Biochem. Pharmacol.* 40: 2353-2362.
[28] Harmon, B. V., Y. S. Takano, C. M. Winterford, and G. C. Gobe (1991) The role of apoptosis in the response of cells and tumours to mild hyperthermia. *Int. J. Radiat. Biol.* 59: 489-501.
[29] Thatte, U. and S. Dahanukar (1997) Apoptosis: clinical relevance and pharmacological manipulation. *Drugs* 54: 511-532.
[30] So, F. V., N. Guthrie, A. F. Chambers, M. Moussa, and K. K. Carroll (1996) Inhibition of human breast cancer cell proliferation and delay of mammary tumorigenesis by flavonoids and citrus juices. *Nutr. Cancer* 26: 167-181.

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