Original Article

Elimination of Enhanced Thermal Resistance of Spheroid Culture Model of Prostate Carcinoma Cell Line by Inhibitors of Hsp70 Induction

Samideh Khoei, Ph.D.[1], Gholam Reza Fazeli, M.Sc.[2], Atefeh Amerizadeh, M.Sc.[2], Delaram Eslimi, M.Sc.[3], Bahram Goliaei, Ph.D.[2]*

1. Medical Physics Department, School of Basic Medical Sciences, Cellular and Molecular Research Center, Iran University of Medical Sciences, Tehran, Iran
2. Laboratory of Biophysics and Molecular Biology, Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran
3. Biology Department, Faculty of Science, Tarbiat Moallem University, Tehran, Iran

**** Corresponding Address: P.O.Box: 13145-1384 , Laboratory of Biophysics and Molecular Biology, Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran***
Emails: goliaei@ibb.ut.ac.ir

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Abstract

Objective: The purpose of this study was to investigate the enhanced thermal resistance mechanism of the DU145 tumor spheroid cultures as compared to the prostate carcinoma cell line's monolayer cultures.

Materials and Methods: DU145 cells were cultured either as spheroids or monolayers. Cultures were treated with hyperthermia in a precision water bath (at 43°C for 60 minutes) and/or quercetin (50 and 500 μM for monolayer and spheroid cultures respectively). After hyperthermic treatment, the cell viability colony forming ability, and the expression of heat shock protein 70 (Hsp70) were examined in both culture systems. Hsp70 expression was studied using the western blot method.

Results: Our results showed that the DU145 monolayer and spheroid cell culture treatment with hyperthermia alone resulted in a marked survival inhibition. Furthermore, the spheroids showed a more significant resistance to hyperthermia compared to the monolayer cultures ($p = 0.01$). They also produced more Hsp70 than the monolayer cultures. Treatment of cells with quercetin reduced the Hsp70 level in both culture systems. However, with the reduced Hsp70 levels, thermal resistance of the spheroids showed a greater decrease in relation to that of the monolayers.

Conclusion: The results suggest that the enhanced hyperthermia resistance mechanism of the spheroid cultures compared to that of the monolayer cultures can be attributed to spheroids' Hsp70 production.

Keywords: Spheroid, Monolayer, Quercetin, Hsp70, Thermal Resistance

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Introduction

Hyperthermia, either alone or in combination with other modalities such as chemotherapy and radiotherapy, is now a well established modality in cancer treatment (1, 2). Physiologic stress, including heat shock, enhances the synthesis of a limited number of intracellular proteins, the so-called heat shock proteins (HSPs) (3). The synthesis of HSPs represents an essential, finely regulated and highly conserved cellular response to adverse environmental conditions which besides heat shock include the presence of amino acid analogues (4), exposure to transition metal ions or sulphydrylic reagents (5), glucose deprivation (6), hypoxia (7), and viral infections (8). Hsp70 has been shown to confer resistance to radiation and hyperthermic treatment for recurrent breast cancer (9). It has also been shown that overexpression of Hsp70 generally confers heat resistance to cancer cells, suggesting that Hsp70, in addition to its functions in the repair and recovery of heat-damaged cells, has a protective function against thermal stress (10, 11). We have reported that DU145 prostate cancer cells in the spheroid cultures showed resistance to hyperthermia at all levels of heat exposure (12). Under the same experimental conditions, spheroids produced higher levels of Hsp70 as compared to monolayer cultures. Therefore, the acquired thermal resistance of spheroid cultures may be attributed to the higher level of Hsp70 production (13). Therfore, downregulation of HSP70 would theoretically be expected to eliminate the thermal sensitivity of spheroid cultures.

It seems that quercetin (3, 3', 4', 5, 7- pentahydroxyflavon), one of the most widely distributed bioflavonoids in the plant kingdom and present in most edible fruits and vegetables, exhibits potent regulatory effects on HSP production (14). It inhibits the growth of malignant cells through several mechanisms such as inhibition of glycolysis (15), enzyme synthesis (16), cell cycle arrest (17) and interaction with type II estrogen binding sites (18).

One effect of quercetin and other flavonoids is to inhibit the activation of HSF1. This in turn leads to the depletion of the strictly HSF1-dependent heat shock protein 72 through inhibition at the transcription level phase (19, 20). It has also been demonstrated that quercetin reduces the heat dependent expression of Hsp70 (21, 22) and it downregulates heat shock transcription factors 1 and 2 in various cells (20, 23).

In the presented study, we have investigated the combination effect of quercetin and hyperthermia (43°C, 1 hour) on the expression of Hsp70 and thermal sensitivity in tumor spheroid and monolayer models of the DU145 prostate carcinoma cell line (24). By reducing the Hsp70 level of spheroid cultures via the administration of higher doses of quercetin, we have shown that the thermal resistance of spheroid cultures reduced to the same level as that of monolayer cultures. DU145 is an established cell line that can self-assemble into large, stable spheroids through a combination of intercellular communication and diffusion (25).

Materials and Methods

Cell line

Human prostate carcinoma cell line DU145 was purchased from Pasteur Institute of Iran. This cell line was maintained in RPMI 1640 (Gibco) supplemented with 10% fetal calf serum (FCS) (obtained locally), 300 U/ml of penicillin (Sigma Aldrich) and 200 mg/L of streptomycin (JABER-EBN-HAYAN).

Monolayer culture

DU145 cells were cultured as a monolayer at a density of 10^4 cells/cm^2 in T-25 tissue culture flasks (Nunc). The cultures were maintained at 37°C in a humidified atmosphere of 7.5% CO_2. Cultures were propagated or cells were harvested by trypsinzing cultures with 0.5 mM EDTA/ 0.25% (w/v) trypsin in phosphate buffer saline (PBS).

Spheroid culture

Spheroids were initiated using the liquid overlay technique (26).

5×10^5 cells were seeded into 100 mm Petri dishes coated with a thin layer of 1% Bacto Agar (Difco Laboratories) with 10 ml of RPMI 1640 supplemented with 10% FCS. The plates were incubated in a 37°C humidified atmosphere with 7.5% CO_2. Half of the culture medium was replaced with fresh medium twice per week.

Heat and quercetin treatment of monolayer cultures

5×10^5 cells per flask were cultured in T-25 culture flasks containing RPMI-1640 culture medium supplemented with 10% FCS for monolayer culture. After 48 hours, they were treated with 50 µM quercetin for 6 hours. The monolayer cells were then exposed to 60 minutes of hyperthermia at 43°C in a precision water bath (Haake F3) with ± 0.1°C accuracy; control cells were exposed to 37°C. They were then harvested, counted and tested for viability and assayed for colony forming ability. Half of the colonies were counted on day 9 and processed for protein analysis as described below. In all experiments, quercetin was dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO added to medium was 0.25% (v/v). The same concentration of DMSO was added to the control cultures.

Heat and quercetin treatment of spheroid cultures

To study the effect of hyperthermia and quercetin on spheroids, 5×10^5 cells were cultured per 100 mm Petri dish coated with a thin layer of 1% agar for multicellular spheroid formation. On day 11, spheroids with mean diameter of 100 µm were treated with 500 µM quercetin and hyperthermia as described for monolayer cultures. They were then treated with 300 µl of PBS containing 1 mM EDTA/0.25% (w/v) trypsin for 10 minutes at 37°C. Trypsin was neutralized by the addition of 700 µl culture medium containing FCS. Half of the spheroids were mechanically disaggregated, and single cells were counted and tested for viability. The cells were then assayed for colony formation ability. The other half were processed for protein analysis as described below.

Trypan blue exclusion assay

A suspension of treated and control single cells from either monolayer or spheroid cultures were mixed with trypan blue at a 9:1 ratio . The resulting mixture was examined within 3-5 minutes under a light microscope (Leica DMLS) and blue colored cells were considered dead. The ratio of unstained

cells to total number of cells was reported as the viability percentage for each cell category.

Clonogenic assay
Treated and control single cell suspensions from either monolayer or spheroid cultures were seeded in 60 mm Petri dishes (Nunc) and grown in RPMI 1640 supplemented with 10% FCS. The cells were incubated at 37°C in a humidified atmosphere of 7.5% CO_2 for ten days. The colonies were counted using an inverted phase microscope (Zeiss Axiovert 405 M) on day 9 and the plating efficiency (PE) was determined.

Survival curves
Survival curves were generated by plotting the log of ratio of the number of colonies formed to the number of colonies produced by related control cells under the heat treatment conditions versus the duration of heat exposure at the given temperature.

Protein isolation and analysis
Cells were washed with PBS and lysed at 4°C for 30 minutes in 100-200 µl of lysis buffer (10 mM Tris-HCl (pH 6.8), 100 µg/ml phenylmethylsulfonyl fluoride (PMSF), 0.14 M NaCl, 1.5 mM $MgCl_2$ and 0.5% NP-40) per 100 mm dish. The lysate was then centrifuged at 12000 rpm for 20 minutes to pellet large cellular debris. Protein concentrations were measured using the Bradford protein assay (27). Equal amounts of samples (50 µl) were mixed with an equal volume of SDS-PAGE loading buffer, heated at 90°C for 5 min, then loaded onto 10% SDS-PAGE gel for electrophoresis.

Western-blot analysis
To perform the Western-blot analysis, the separated proteins were electroblotted onto nitrocellulose membranes (Schleider & Schuell), and then probed with monoclonal anti-heat shock protein 70 (anti-Hsp70) (Sigma Aldrich). The primary antibody was detected with secondary anti-rabbit IgG conjugated to horseradish peroxidase to generate a 4-chloro-1-naphtol signal (Sigma Aldrich). To help in identification, protein standard of recombinant rat Hsp70 protein (Stressgen) was included in the 10% SDS-PAGE gel. The protein standard was visible on the Western-blot nitrocellulose membrane. The antibody labeled Hsp70 polypeptide band images on the developed blots were digitally captured using a UVItec gel documentation system and band intensities were measured using the TotalLab electrophoresis analysis software.

Statistical analysis
Data for figures 1 and 2 were given as mean values± SEM (standard error of the mean) of 3 experiments. Student's t test was applied as appropriate. A value of $p \leq 0.05$ was considered significant.

Results

Cell characteristics
The DU145 prostate carcinoma cell line grows as monolayer on plastic culture flasks with a population doubling time of approximately 23 hrs. These cells can survive in low population densities and form colonies with at least 50 cells within 9 days. The DU145 cells are also able to form spheroids in liquid overlay cultures.

Effect of hyperthermia and quercetin on cell viability
Immediately after cell treatment with quercetin and hyperthermia, monolayer and spheroid cells were dispersed to single cells. They were then counted and viability was determined using the trypan blue dye exclusion assay. Table 1 and figures 1A and 1B show the effect of quercetin and hyperthermia on the viability of DU145 cells from monolayer and spheroid cultures respectively.

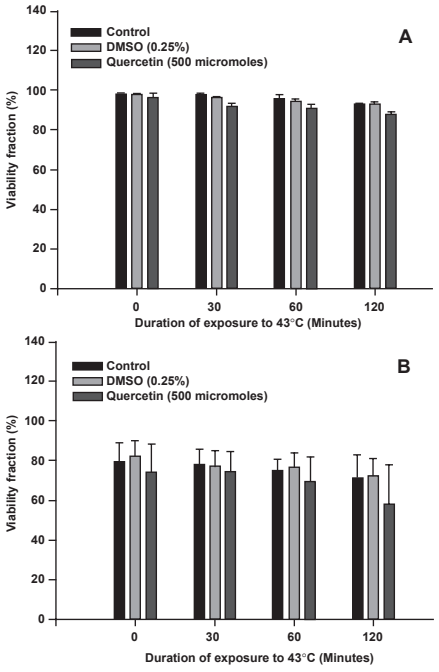

Fig 1: Effects of quercetin and hyperthermia on cell viability. A) Effects of quercetin (50 µM) and/or hyperthermia at 43°C for various heating durations on the viability of DU145 monolayer culture cells. B) Effects of quercetin (500 µM) and/or hyperthermia at 43°C for various heating durations on the viability of DU145 spheroid culture cells.

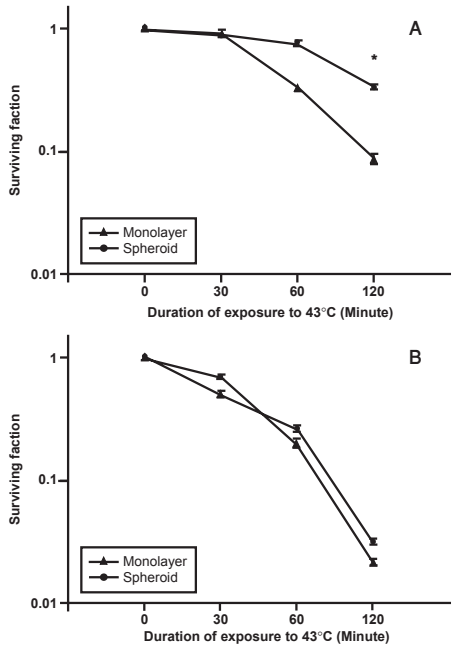

Fig 2: Effects of quercetin and hyperthermia on cell surviving fraction (colony forming ability) of DU145 cells. A. Effects of hyperthermia at 43ºC for various durations on monolayer and spheroid cultures. (*p = 0.01). B. Effects of hyperthermia at 43ºC for various durations, quercetin (50 µM) on monolayer and hyperthermia at 43ºC for various durations, and quercetin (500 µM) on spheroid culture cell surviving fraction.

As can be seen in both figures, hyperthermia at 43°C, alone and in combination with DMSO diluent did not have any effect on the viability of cells in either culture. Treatment with the combination of hyperthermia and quercetin markedly reduced cell viability in both culture types.

Effect of hyperthermia and quercetin on colony forming ability

The cell response to hyperthermia and quercetin in terms of colony formation was studied by applying quercetin plus hyperthermia at 43°C for various periods of time. Plots of survival fraction versus heating duration at 43°C alone and in combination with quercetin for monolayer and 11 days old spheroid cultures are shown in tables and figures 2A and B, respectively. Table and figure 2A clearly show that treatment of DU145 monolayer and spheroid cells with hyperthermia alone resulted in a marked inhibition of survival, and that the spheroids showed significantly higher resistance to hyperthermia as compared to monolayers (*p = 0.01). Combined treatment of DU145 cells with hyperthermia and 50 µM of quercetin in monolayer cultures reduced the cultures' resistance to hyperthermia (compare tables and figures 2A and B). Also, combined treatment of spheroid cultures with hyperthermia and 500 µM of quercetin reduced their hyperthermia resistance level to that of monolayers (Table and Fig 2B).

Table 1: Effects of quercetin and/or hyperthermia at 43ºC for various heating durations on the viability of monolayer and spheroid cultures of DU145 cells.

Sample			Viability ± SD	Viability ± SD
Temperature (°C)	**Heating Duration (minute)**	**Drug**	**Monolayer culture**	**Spheroid culture**
37	0	-	77.1 ± 17.11	99.37 ± 0.77
37	0	DMSO	83.05 ± 9.97	99.02 ± 1.01
37	0	Quercetin	74.00 ± 19.79	98.17 ± 2.03
43	0	-	79.60 ± 13.57	98.25 ± 0.75
43	0	DMSO	82.55 ± 10.67	97.85 ± 0.98
43	0	Quercetin	74.30 ± 20.22	96.57 ± 2.62
43	30	-	77.95 ± 11.25	98.00 ± 0.66
43	30	DMSO	77.55 ± 10.67	96.72 ± 1.00
43	30	Quercetin	74.90 ± 14.00	92.70 ± 2.87
43	60	-	75.40 ± 7.63	95.90 ± 3.03
43	60	DMSO	77.05 ± 9.97	95.35 ± 2.37
43	60	Quercetin	68.95 ± 18.31	91.7 ± 3.31
43	120	-	71.45 ± 16.19	93.57 ± 1.03
43	120	DMSO	72.75 ± 12.37	93.42 ± 1.50
43	120	Quercetin	58.05 ± 28.35	88.4 ± 1.99

Effect of hyperthermia and quercetin on Hsp70 level

To investigate the effect of quercetin and hyperthermia on Hsp70 levels, cells from the monolayer and spheroid cultures were treated as described above. Hsp70 levels were measured during the first 7 hurs after treatment. Western-blot results showed that 7 hurs after heat treatment, heat shocked DU145 spheroid cells produced Hsp70. DMSO diluent had no effect on Hsp70 synthesis. Quercetin was found to inhibit Hsp70 synthesis. As can be seen in figures 3-6, Hsp70 induction was reduced in the monolayer and spheroid culture cells thru exposure to 50 µM and 500 µM quercetin respectively.

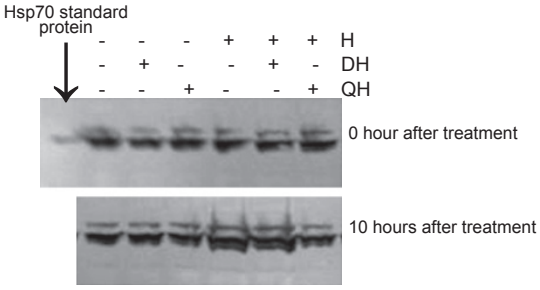

Fig 3: Effects of quercetin (50 µM) and/or hyperthermia (1 hour at 43ºC) on Hsp70 level in monolayer culture of DU145 carcinoma cell line using the Western-blot method. H: DU145 cells exposed to heat shock at 43ºC for 1 hour; QH: cells pretreated with quercetin for 6 hour at 37ºC, then exposed to hyperthermia and incubated for 10 hours at 37ºC; DH: cells pretreated with DMSO diluent (0.25%) for 6h followed by hyperthermia and incubation for 10 hours at 37ºC.

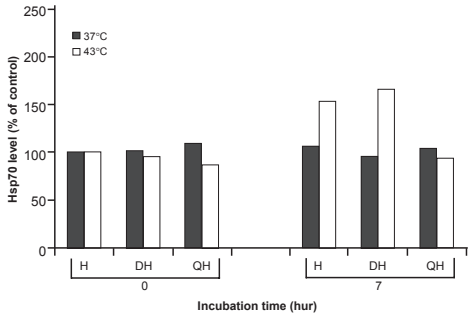

Fig 4: Results of scanning of bands in figure 3 using the TotalLab electrophoresis analysis program. H: DU145 cells exposed to heat shock at 43ºC for 1 hour; QH: cells pretreated with quercetin for 6 hour at 37ºC, then exposed to hyperthermia and incubated for 10 hours at 37ºC; DH: cells pretreated with DMSO diluent (0.25%) for 6 hour followed by hyperthermia and incubation for 10 hours at 37ºC. Y-axis indicates band intensities defined by the number of pixels under each peak.

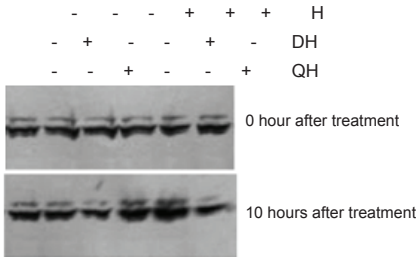

Fig 5: Effect of quercetin (500 µM) and/or hyperthermia (1 hour at 43ºC) on Hsp70 level in day 11 of spheroid cultures using the Western-blot method. H: DU145 cells exposed to heat shock at 43ºC for 1 hour; QH: cells pretreated with quercetin for 6 hours at 37ºC, then exposed to hyperthermia and incubated for 10 hours at 37ºC; DH: cells pretreated with DMSO diluent (0.25%) for 6 hours followed by hyperthermia and incubation for 10 hours at 37ºC.

Table 2A: Effects of hyperthermia at 43ºC for various durations on monolayer and spheroid cultures

Monolayer		Spheroid	
Heating duration (minute)	**Survival fraction ± SD**	**Heating duration (minute)**	**Survival fraction ± SD**
0	1 ± 0	0	1 ± 0
30	0.91 ± 0.05	30	0.87 ± 0.01
60	0.32 ± 0	60	0.75 ± 0.04
120	0.08 ± 0.01	120	0.33 ± 0.01

Table 2B: Effects of hyperthermia at 43ºC for various durations and quercetin (50 µM) on monolayer culture cells, and hyperthermia at 43ºC for various durations and quercetin (500 µM) on spheroid culture cells

Monolayer		Spheroid	
Heating duration (minute)	**Survival fraction ± SD**	**Heating duration (minute)**	**Survival fraction ± SD**
0	1 ± 0	0	1 ± 0
30	0.72 ± 0.04	30	0.50 ± 0.03
60	0.18 ± 0.02	60	0.26 ± 0.02
120	0.02 ± 0.001	120	0.03 ± 0.01

Quercetin was found to inhibit Hsp70 synthesis. As can be seen in figures 3-6, Hsp70 induction was reduced in the monolayer and spheroid culture cells thru exposure to 50 µM and 500 µM quercetin respectively.

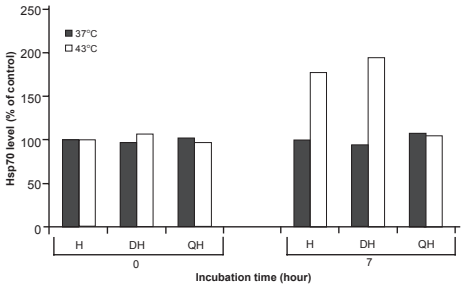

Fig 6: Results of scanning of bands in figure 5 using the TotalLab electrophoresis analysis program. H: DU145 cells exposed to heat shock at 43ºC for 1 hour; QH: cells pretreated with quercetin for 6 hours at 37ºC, then exposed to hyperthermia and incubated for 10 hours at 37ºC; DH: cells pretreated with DMSO diluent (0.25%) for 6 hours followed by hyperthermia and subsequent incubation for 10 hours at 37ºC. Y-axis indicates band intensities defined by the number of pixels under each peak.

Discussion

For many years, cell biologists have investigated cancer primarily by using monolayer-grown stabilized tumor cell lines. Although, this in vitro model has yielded much valuable information regarding the mechanisms at the basis of malignant growth, it is nonetheless an unsuitable representation of in vivo tumors. It should be noted that solid tumors grow in a three-dimensional spatial array and that their cells are exposed to non-uniform oxygen and nutrient distributions as well as other physical and chemical stresses (28, 29). Thus, in an attempt to design more suitable in vitro models which take into consideration the three-dimensional arrangement of solid tumors, multicellular tumor spheroids have been developed (30). Although research on these models may be experimentally quite complex, every effort should be made to conduct such investigations in order to obtain more precise information regarding the effects of antineoplastic agents and hyperthermia on tumor cell death, and the mechanisms responsible for tumor cell resistance. The aim of this study was to investigate the relationship between the thermoresistance properties of human prostate cell line spheroids and monolayers with the expression of Hsp70 using an Hsp70 induction inhibitor agent. The clinical application of hyperthermia thru increase of tissue temperatures (40-44°C range) has been integrated in multimodal anti-cancer strategies (31). Hyperthermia kills cells largely through the induction of lethal protein aggregation and denaturation cascades, and is antagonized by HSP activity which leads to protein refolding (32). It has been reported that Hsp70 is involved in protecting cells against lethal heat treatment (33) and that the mechanism of heat-induced cell killing was primarily apoptosis, which was protected by overexpression of the Hsp70 gene (34, 35). Therefore, HSPs might be responsible for the protection against heat-induced apoptosis.

Our previous study showed that spheroid formation was not a stress generating process compared to monolayer culture cell formation. Cells in spheroid cultures produced nearly the same amount of Hsp70 during the 26 days of study. They also showed resistance to hyperthermia at all levels of exposure (12). Under the same experimental conditions, spheroids produced higher levels of Hsp70 as compared to monolayer cultures. Therefore, we concluded that the acquired thermal resistance of spheroid cultures may be attributed to their increased Hsp70 production (13). Hence low Hsp70 inducibility and a low level of Hsp70 synthesis may increase cell thermal sensitivity. Quercetin, a synthetic flavonoid, has been shown to inhibit stress response and especially inhibit (verb agreement) Hsp70 synthesis through the inhibition of heat-shock-transcription factor 1 or 2 (20, 22, 23). Several reports have shown that quercetin significantly increases heat- induced apoptotic cell death by inhibiting Hsp70 synthesis (36-38). Our results presented here support this hypothesis. Results described in the presented study show that the total Hsp70 level-increase as a result of heating at 43ºC for 1h was inhibited by quercetin in both models of spheroid and monolayer cultures (Figs. 3-6). A comparison of figures 2A and 2B indicates that quercetin reduced the thermal resistance of cells cultured in monolayer. This emphasizes the critical role of Hsp70 in protecting cells against hyperthermia-induced stress. By using a higher concentration of quercetin in spheroid cultures, Hsp70 levels were reduced to that of monolayer cultures. This in return, resulted in a similar thermal resistance of cells in spheroid and monolayer cultures (Fig 2B). This reduction is not due to the change of viability of cells in these two culture models (Fig 1). As can be seen, the concentration of quercetin used for Hsp70 inhibition in spheroid cultures was ten times greater than that used for monolayer cultures. The use of two different concentrations of

quercetin in these two culture models was due to differential chemoresistance of cells in the two spheroid and monolayer culture models.

Conclusion

Our results emphasized our previous conclusion that the acquired thermal resistance of spheroid cultures compared to monolayer cultures attributed to their higher level of Hsp70 production. Therefore, by using a higher concentration of an Hsp70 induction inhibitor, thermal resistance of the spheroids decreased to the level of monolayer cultures. Quercetin is a water insoluble material that cannot be dissolved in culture medium. For this reason, its transport across the cell membrane and transfer into the cells is very difficult. Hence, our suggestion for the next study is the use of copolymeric nanoparticles as carriers for the proper drug-dosage transport into cells.

Acknowledgments

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