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Controlled Molecular Targeting of Inducible Heat Shock Proteins

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Abstract

In an effort to devise schemes to site-specifically deliver drugs "on demand" we have leveraged the inducible expression properties of the well-characterized class of stress-response factors, the heat shock proteins. We asked whether a small molecule ligand selective for such proteins could be used as a potential theranostic platform. By conjugating a fluorescent dye to a recently identified nucleoside analog with modest affinity for heat shock protein 70 (HSP70), we have found that cellular uptake can be enhanced following transient heat shock in vitro. The in vivo performance of this compound was further evaluated in nude mice subjected to locoregional hyperthermia which showed a modest level of accumulation in the treated muscle. This effect correlates with relative levels of HSP70 over expression in the treated tissue, supporting the hypothesis that molecular targeting can be enhanced by inducing endogenous proteins through external means.

Keywords: Heat shock proteins; HSP70; Targeted drug delivery; Molecular targeting; Controlled drug delivery; Hyperthermia

Introduction

The ability to control the site-specific delivery of drugs on demand may circumvent many of the pharmacological dilemmas in achieving desired cytotoxicity to malignant tumors or sites of diseased tissue versus reducing systemic toxicity. Given the relatively nonselective nature of most anticancer agents, the therapeutic index is often narrow for otherwise highly potent drugs. We have approached this problem by postulating that drug delivery may be better controlled if specific targets could be reliably altered by external means. Ideally, such an approach would require methods that were both noninvasive and inconsequential in terms of locoregional or systemic adverse effects. Capitalizing on known physical methods for inducing localized hyperthermia, for example through the use of focused ultrasound [1,2], we sought to identify suitable endogenous targets that could be highly overexpressed. This notion prompted us to hypothesize that the class of well-characterized heat shock proteins (HSP) may be used as key targets to enhance intracellular drug delivery. Our approach was to use a high affinity ligand for such inducible proteins as a targeting moiety conjugated to another compound which may be an imaging molecule (or radionuclide) or cytotoxic compound, to form a suitable HSP-targeted drug complex. Ideally, the targeting moiety alone would be nontoxic and amenable to further modifications that still permit cellular permeability. Unfortunately, HSP-specific pharmacological agents reported in the literature were both sparse and suboptimal with regard to protein affinity, pharmacokinetics, and/or toxicity. Among the few HSP-specific agents, a series of small molecules based on a nucleoside backbone reported by Williamson et al. [3] offered a potential starting point to test our hypothesis.

Leveraging HSPs for enhancing targeted drug delivery—Heat shock proteins are phylogenetically conserved gene products that catalyze the proper refolding of client proteins presumably altered during heat denaturation or other physicochemical events, collectively described as "stress" [4-8]. Proteins require the proper conformation and the action of HSPs are essential for survival during environmental changes as well as in maintaining the normal biochemical activities of the cell. However these pro-survival functions of HSPs appear to have been usurped by malignant cells which render these gene products as potentially attractive pharmacologic targets [9-12]. HSPs have been implicated in tumor cell proliferation, differentiation, invasion, and metastasis with important clinical consequences for cancer therapy [10,12-14]. Among the well-characterized HSPs, the 70 kDa group collectively termed "HSP70," has been shown to exert strong cytoprotective effects by limiting aggregation of intracellular proteins and directly antagonizing apoptosis pathways [13,15-17]. Concomitant with increased cellular proliferation rate, high baseline levels of HSP70 expression have been observed in a variety of human tumors [18-22]. Interestingly, levels of HSP70 expression correlates with poor clinical prognosis in breast, endometrial, cervical, and bladder carcinomas and is implicated in the development of drug resistance [10].

Structural analysis of HSP70 has revealed that the N-terminus ATPase domain is involved in substrate engagement and protein refolding [23]. Using this domain as a potential pharmacologic target, Williamson et al. [3] synthesized several adenosine analogs and found a few derivatives to bind HSP70 in vitro in the low micromolar range. Using these compounds as a starting point for producing HSP70-selective ligands, we set out to test our hypothesis for enhancing the intracellular targeting of an inducible protein on demand. In this report we describe our findings using a selected adenosine analog conjugated to the commonly used fluorophore, 6-carboxyfluorescein, that serve both as an imaging moiety and a mock "drug" both in cultured cells and in live mice.

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Materials and Methods

Synthesis of adenosine analogs

Adenosine-derived HSP70 inhibitor-12 (AD12) was synthesized according to a previously published procedure with some modifications to reaction conditions [3]. Briefly, the synthetic scheme for AD12-FAM is illustrated in Figure 1. Compound 1 was prepared by alkylation with the corresponding halide in the presence of sodium hydride in N,N-dimethylformamide (DMF) at room temperature. Compound 1 was purified by column chromatography and isolated in 85% yield. The 6-Cl of the compound 1 was converted to compound 2 by treatment with 5N ammonium hydroxide solution in ethanol through microwaves at 115°C for 1hr. It was purified by silica gel column and isolated in 76% yield. Compound 2, which selectively underwent bromination at the 8-position upon treatment with bromine in dioxane and 5% aqueous solution of Na_2HPO_4 at room temperature to obtained compound 3, which was purified by silica gel column and isolated in 79% yield. Compound 4 was prepared by reaction of the corresponding amine, 3,4-dichlorobenzylamine in ethanolic solution at 130°C for 1.5h through microwaves. Purified yield in this step was 77%. The deprotection of acetonide moiety of 4, was using trifluoroacetic acid in water at room temperature and also heated at 50°C for 30 min. It was purified by column chromatography and afforded 5 in 79% yields. Conjugate 6, (AD12-FAM) was obtained by the reaction of 5 (AD12), with 6-carboxyfluorescein N-hydroxysuccinimide ester (6-FAM-NHS) in DMF in the presence of triethylamine (pH, 8.2-9.0). The reaction mixture was stirred in the dark at ambient temperature for overnight and quenched by 5% acetic acid in water to make neutralization. The crude conjugate 6 was purified by semi preparative reversed-phase HPLC and isolated in 65% yield. Samples were dissolved in phosphate buffered saline solution, pH 7.4 (PBS) and 5% DMSO (volume/volume) and filter sterilized using a 0.2 micron mini-syringe filter (Whatman) prior to use.

Cultured cells heat shock procedures

M21 human melanoma cell cells (courtesy of Dr. D. Cheresh, U.C. San Diego) were grown in a 24-well plate at a density of 400,000 cell/well using RPMI-1640 media supplemented with 10% FBS and 50 U/ml of penicillin and 50ug/ml streptomycin in a total of 2 ml. They were allowed to attach for 24 hrs prior to heat shock. Cells were heated at 43°C for 1 hr in a humidified incubator with 5% CO_2 and allowed to recover at 37°C for 5 hours. After the recovery period compounds at various concentrations were added directly to the media and incubated for 1hr at 37°C. Cells were then washed 3 times with prewarmed PBS (37°C), followed by scrap harvesting, counted and diluted to 100,000 cells/ml. Approximately 10,000 cells per well were seeded in 96-well black plates and measured in a fluorometric plate reader (FLUOstar OPTIMA, Durham, NC) with a 485/520 nm filter set. For each sample tested a total of 6 runs were assayed.

Western blotting and immunohistochemistry

Control and heat-shock treated cells were washed twice with PBS and lysed in lysis buffer (Cell signaling, Cat. #9803S) on ice for 1 hr. Total protein concentrations were measured with Advanced Protein Assay Reagent (Cytoskeleton, Cat. #ADV01). Cell lysates were boiled in 5X sample buffer and loaded on the 4-20% Precise Protein Gels

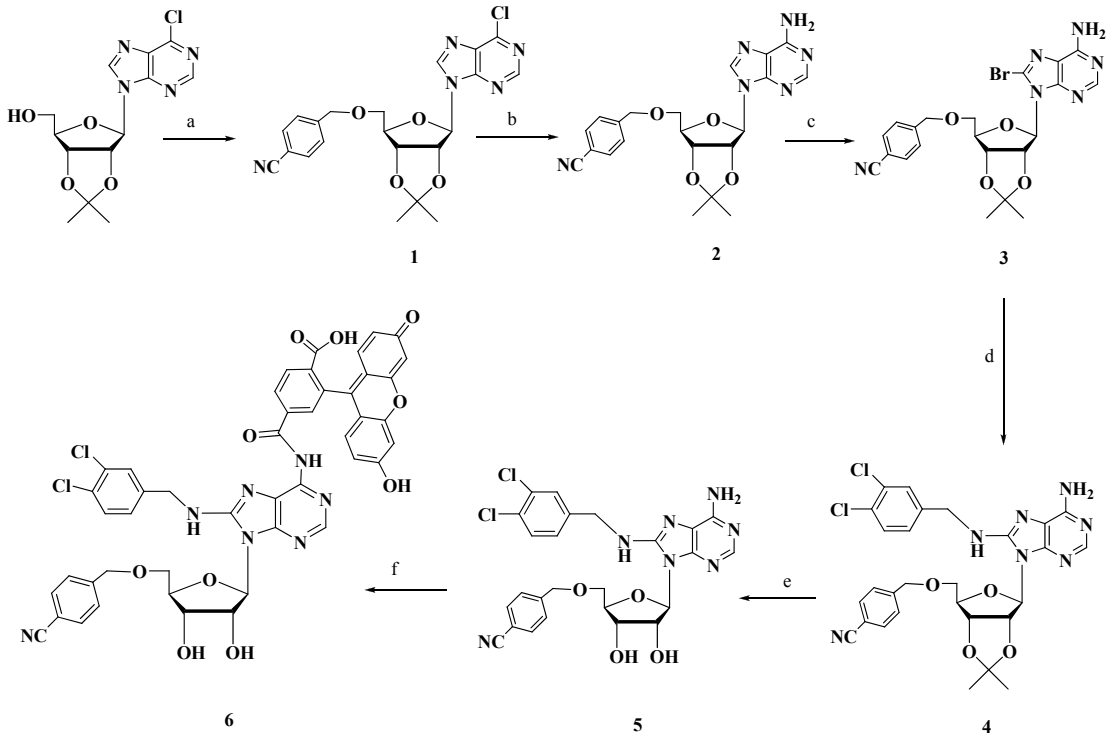

Figure 1: Synthetic scheme of AD12-FAM. Reagents and reaction conditions: (a) R_1, DMF, NaH, r.t., 1 hr, 89%; (b) NH_4OH, EtOH, 115° C, microwave, 1 hr, 76%; (c) Br_2, Na_2HPO_4(aq), dioxane, r.t., 1 hr, 79%; (d) R_2, EtOH, 130° C, microwave, 1.5 hr, 77%; (e) H_2O, TFA, r.t. (30 mim), 50° C (30 min), 79%; (f) FAM-NHS, DMF, Et_3N, r.t., overnight, HOAc, 65%.

(Thermo Scientific, Cat. 25204). All the samples were transferred onto the PVDF membrane (BioRad, Cat. #162-0177) at 40 V for 2 hrs and blocked with blocking buffer (5% non-fat milk in TBS(25 mM Tris, pH 7.4; 130 mM NaCl; 2.7 mM KCl)) for 2 hrs at room temperature. The membrane was incubated at 4°C overnight in blocking buffer containing primary antibody against either HSP70 (R&D Systems, Cat. MAB1663, 1:2500 dilution) or β-Actin (Sigma-Aldrich, Cat. A1978, 1:3000 dilution). After each primary antibody binding, the membrane was washed three times every 10 min in TBST (TBS with 0.1% Tween-20). The membrane was incubated with anti-mouse Ig, horseradish peroxidase linked (GE Healthcare, Cat. NXA931, 1:5000 dilution) for 90 min in room temperature. After washing three times, luminescence of the blot was captured using an ImageQuant LAS4000 (GE Healthcare). HSP70 detection was also performed in cell lines from human and rat sources including HCT116 (human), Jurkat (human), MDA-MB231 (human), and C6 (rat glioma). Harvested mouse muscle samples were fixed in 10% buffered formalin solution and paraffin-embedded for immunohistochemistry (IHC) using the same anti-HSP70 monoclonal antibody and counterstaining with hematoxylin. IHC procedure was performed by the pathology core service facility of The Methodist Hospital.

In vivo imaging in mice

Localized heating was performed in the right hind limb of isoflurane (2%) anesthetized nude mice (female nu/nu, approximately 25 g in weight) using a water bath set at 47°C for 4.5 minutes. Following 5 hours of recovery, 130 µL AD12-FAM (10 mM) was administered by tail vein injection followed by fluorescence imaging at multiple time points up to 2 hours post injection using a CRi Maestro multispectral imaging system (Caliper Life Sciences) and using an appropriate filter set (excitation/emission = 445-490/515nm) and 2 x 2 binning. Mice were anesthetized with 2% isoflurane during the live imaging acquisitions. At 2 hours post injection, mice were sacrificed using CO_2 gas and thereafter the skin was regionally removed and subjects were re-imaged. Relative fluorescence signal was measured with manually drawn region of interest (ROI) using the dedicated analysis software. All animal experiments were performed in strict compliance with a protocol approved by the Institutional Animal Care and Use Committee of the Methodist Hospital Research Institute.

Results and Discussion

Heat shock increases the cellular uptake of AD12-FAM in cultured cells— The specific adenosine derivative, AD12, as originally reported by Williamson et al. [3] was conjugated to 6-carboxyfluorescein yielding AD12-FAM (Figure 1) and tested for cellular uptake in a human cell line of malignant melanoma (M21) subjected to transient heat shock. The procedure was performed under aseptic conditions in a tissue culture incubator. As shown in Figure 2, cellular uptake of AD12-FAM is modestly increased following heat treatment only at the highest concentration tested (20 µM). Lower concentrations of this compound did not result in any significant increase relative to the non-heat shocked control cells. This effect is not surprising given the relatively low affinity since the K_D of the parent adenosine analog compound was measured to be 0.3 µM [3]. Nonetheless these results indicate that this fairly large compound (FW 985 Da) is capable of intracellular translocation through a mechanism that remains to be elucidated. Controls using 6-carboxyfluorescein (FAM) alone at the same molar concentrations showed no significant cellular uptake (data not shown). Western blot analysis of HSP70 expression showed a relatively high level of baseline HSP70 expression in exponentially growing M21 cells which mildly increased following heat shock (data not shown). However this effect did not appear to directly correspond with the observed levels of AD12-FAM uptake in heat-exposed M21 cells, suggesting that this compound may also target other HSP class members that are likely to be over-expressed. This possibility is plausible since the adenosine analog targets the ATPase domain which are structurally similar for several HSP70 isoforms [24] .Interestingly, we have found that other cell lines tested for baseline expression of HSP70 was also variably high (data not shown), consistent with other reports in the literature. These in vitro results prompted us to evaluate the vivo uptake of this compound by testing for accumulation in normal tissue subjected to localized hyperthermia.

Enhancement of AD12-FAM accumulation in normal tissue treated by localized hyperthermia—Although the cell culture studies indicated that a high concentration of AD12-FAM was needed to observe relatively modest levels of increased uptake, this system may not accurately reflect the physiological status of inducible HSP70 in normal tissues. Hence we asked if hyperthermia directed to a specific site in a live organism may result in a more robust heat shock response that could be detected by noninvasive imaging. We approached this question by using a mouse model that was treated with localized hyperthermia using a water bath apparatus devised for this purpose. Similar to the experimental conditions used in the cultured cell experiments we selected a time point to optimize the detection of overexpressed HSP70. This was empirically determined to be approximately 5 to 6 hours, consistent with previously published results [25]. Following mild heat treatment of the right posterior limb of a nude mouse, approximately 1.3 µg of either 6-carboxyfluorescein (FAM) or AD12-FAM was intravenously administered via the tail vein. Animals were imaged using a murine-dedicated fluorescence imaging system at 30 minutes and 2 hours post injection. As shown in the Figure 3, live animals injected with

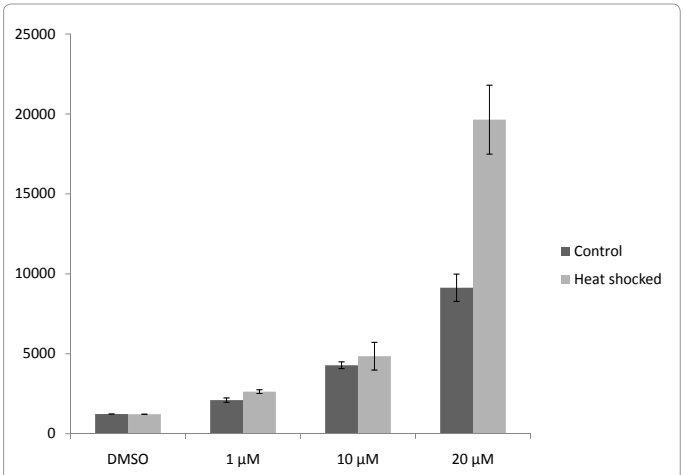

Figure 2: Enhanced uptake of AD12-FAM in cultured human cells (melanoma) subjected to heat shock. Human melanoma cells were grown at 37° C (dark gray) or heat shocked at 43° C for 1 hr in a tissue culture incubator (light gray) and allowed to recover for 5 hrs. AD12-FAM was added to the culture media and allowed to incubate for 1 hr at 37° C, followed by cell harvest and fluorescence measurements. Relative fluorescence values were plotted for the vehicle (DMSO) and AD12-FAM at three final concentrations: 1, 10, and 20 µM. Error bars indicate standard error of the mean for 6 runs.

the fluorescent dye alone and imaged at 2 hours demonstrated no significant fluorescence signal in the heat-treated limb (indicated with an arrow) (Figure 3A). However, administration of AD12-FAM resulted in mild fluorescence signal in the treated limb (Figure 3C). Observation of the contralateral sides for either mouse reveals no significant amount of detectable signal. Since 6-carboxyfluorescein has limited sensitivity in vivo due to soft tissue attenuation of the excitation and emission light wavelengths, the mice were euthanized immediately after the imaging at 2 hours, and the regional skin was removed to expose the underlying muscle tissue followed by repeat imaging. For the 6-carboxyfluorescein subject, no significant fluorescence signal was detected; however the AD12-FAM signal was substantially higher (compare Figure 3B and 3D). For both mice, the signal in the exposed muscles on the contralateral (untreated) limbs demonstrated no significant fluorescence signal (data not shown). Fluorescence signals of the treated tissue were quantified using manually drawn regions of interest and the intensities plotted in Figure 3E. Fluorescence signal at 30 minutes post injection of either FAM or AD12-FAM was overall higher however there is nearly complete "washout" of signal from the FAM control at 2 hours. Signal from the contralateral, unheated limbs, were similar to the FAM treated side at both time points (data not shown). These initial findings indicate that the pharmacokinetic properties of AD12-FAM result in detectable levels of accumulation within locally heated tissue which correlates with HSP70 overexpression (Figure 4). Repeat trials of localized hyperthermia treatment showed considerable variability in observed AD12-FAM uptake; however this

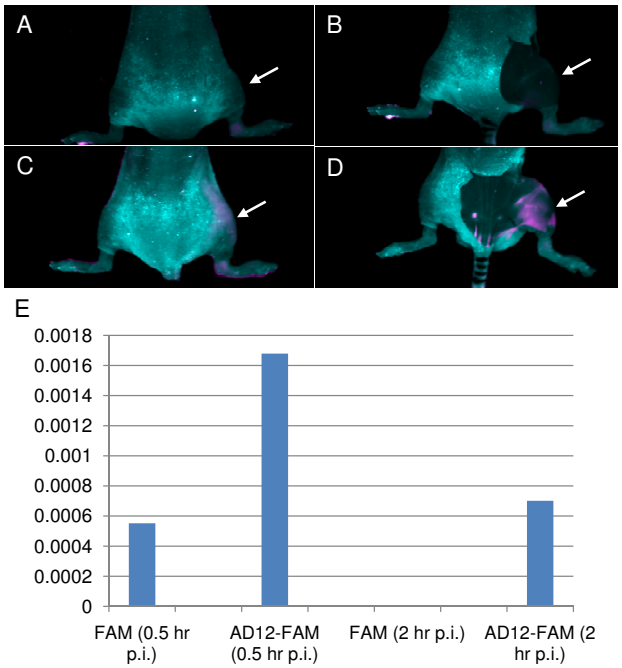

Figure 3: *In vivo* imaging of local hyperthermia treatment in nude mice. Nude mice were locally treated with mild hyperthermia to the right posterior limb and following a recovery period of 5 hours, 6-carboxyfluorescein (FAM) (A, B) or AD12-FAM (C, D) were intravenously administered and imaged at 2 hrs using a fluorescence camera with appropriate excitation/emission filters. Immediately after this imaging, mice were euthanized and the skin removed to expose the underlying muscle (B and D). Arrow indicates the treated limbs. Regions of interest were drawn around the heat-treated limb for each of the 6-carboxyflurescein and AD12-FAM mouse subjects and fluorescence signal quantified at 30 minutes and 2 hrs post injection (p.i.) of live, intact, mice (E).

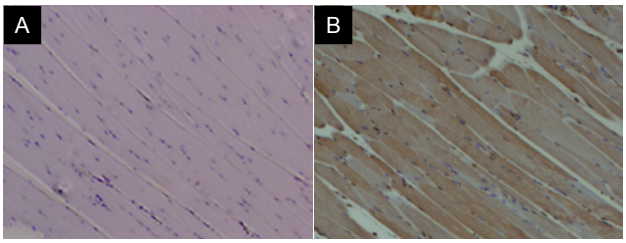

Figure 4: HSP70 is overexpressed in heat-treated muscle. Muscle from nude mice treated with mild hyperthermia were fixed and processed for chromogenic immunohistochemistry using an anti-HSP monoclonal antibody and counter stained with hematoxylin. Untreated (A) and heat-treated muscle (B) harvested from the mouse treated with AD12-FAM. (20X optical).

effect appeared to correlate with levels of HSP70 expression which also was variable as determined by immunohistochemical analysis (data not shown). Although the mechanism for this variability remains poorly explained, changes in HSP70 expression have been implicated with certain gas anesthetics including isoflurane which was also used in the current work [26]. Furthermore, the relatively poor affinity of the adenosine analog used in this study likely contributes to the variable targeting despite the strong in vivo induction of HSP70. Molecular imaging agents with intended pharmacological targeting properties places stringent pharmacokinetics requirements which are not fulfilled in the current study and future refinements to this approach awaits identification of high affinity ligands for inducible targets. Nonetheless, the experimental results presented in this work support the hypothesis that site-directed localization of a composite small molecule may be controllable by the induction of endogenous intracellular targets.

References

1. O'Neill BE, Li KC (2008) Augmentation of targeted delivery with pulsed high intensity focused ultrasound. Int J Hyperthermia 24: 506-520.
2. Hynynen K, Lulu BA (1990) Hyperthermia in cancer treatment. Invest Radiol 25: 824-834.
3. Williamson DS, Borgognoni J, Clay A, Daniels Z, Dokurno P, et al. (2009) Novel adenosine-derived inhibitors of 70 kDa heat shock protein, discovered through structure-based design. J Med Chem 52: 1510-1513.
4. Morimoto RI, Kline MP, Bimston DN, Cotto JJ (1997) The heat-shock response: regulation and function of heat-shock proteins and molecular chaperones. Essays Biochem 32: 17-29.
5. Jaattela M, Wissing D (1992) Emerging role of heat shock proteins in biology and medicine. Ann Med 24: 249-258.
6. Becker J, Craig EA (1994) Heat-shock proteins as molecular chaperones. Eur J Biochem 219: 11-23.
7. Craig EA (1989) Essential roles of 70kDa heat inducible proteins. Bioessays 11: 48-52.
8. Lindquist S, Craig EA (1988) The heat-shock proteins. Annu Rev Genet 22: 631-677.
9. Calderwood SK, Ciocca DR (2008) Heat shock proteins: stress proteins with Janus-like properties in cancer. Int J Hyperthermia 24: 31-39.
10. Ciocca DR, Calderwood SK (2005) Heat shock proteins in cancer: diagnostic, prognostic, predictive, and treatment implications. Cell stress chaperones 10: 86-103.
11. Patury S, Miyata Y, Gestwicki JE (2009) Pharmacological targeting of the Hsp70 chaperone. Curr Top Med Chem 9: 1337-1351.
12. Soti C, Nagy E, Giricz Z, Vigh L, Csermely P, et al. (2005) Heat shock proteins as emerging therapeutic targets. Br J Pharmacol 146: 769-780.

13. Garrido C, Brunet M, Didelot C, Zermati Y, Schmitt E, et al. (2006) Heat shock proteins 27 and 70: anti-apoptotic proteins with tumorigenic properties. Cell cycle 5: 2592-2601.

14. Schmitt E, Gehrmann M, Brunet M, Multhoff G, Garrido C (2007) Intracellular and extracellular functions of heat shock proteins: repercussions in cancer therapy. J Leukoc Biol 81: 15-27.

15. Lanneau D, Brunet M, Frisan E, Solary E, Fontenay M, et al. (2008) Heat shock proteins: essential proteins for apoptosis regulation. J Cell Mol Med 12: 743-761.

16. Parcellier A, Schmitt E, Brunet M, Hammann A, Solary E, et al. (2005) Small heat shock proteins HSP27 and alphaB-crystallin: cytoprotective and oncogenic functions. Antioxid Redox Signal 7: 404-413.

17. Sreedhar AS, Csermely P (2004) Heat shock proteins in the regulation of apoptosis: new strategies in tumor therapy: a comprehensive review. Pharmacol Ther 101: 227-257.

18. Hsu PL, Hsu SM (1998) Abundance of heat shock proteins (hsp89, hsp60, and hsp27) in malignant cells of Hodgkin's disease. Cancer Res 58: 5507-5513.

19. Kocsis J, Madaras B, Toth EK, Fust G, Prohaszka Z (2010) Serum level of soluble 70-kD heat shock protein is associated with high mortality in patients with colorectal cancer without distant metastasis. Cell stress chaperones 15: 143-151.

20. Malusecka E, Zborek A, Krzyzowska-Gruca S, Krawczyk Z (2001) Expression of heat shock proteins HSP70 and HSP27 in primary non-small cell lung carcinomas. An immunohistochemical study. Anticancer Res 21: 1015-1021.

21. Protti MP, Heltai S, Bellone M, Ferrarini M, Manfredi AA, et al. (1994) Constitutive expression of the heat shock protein 72 kDa in human melanoma cells. Cancer Lett 85: 211-216.

22. Rerole AL, Jego G, Garrido C (2011) Hsp70: anti-apoptotic and tumorigenic protein. Methods Mol Biol 787: 205-230.

23. Sondermann H, Scheufler C, Schneider C, Hohfeld J, Hartl FU, et al. (2001) Structure of a Bag/Hsc70 complex: convergent functional evolution of Hsp70 nucleotide exchange factors. Science 291: 1553-1557.

24. Wisniewska M, Karlberg T, Lehtio L, Johansson I, Kotenyova T, et al. (2010) Crystal structures of the ATPase domains of four human Hsp70 isoforms: HSPA1L/Hsp70-hom, HSPA2/Hsp70-2, HSPA6/Hsp70B', and HSPA5/BiP/GRP78. PLoS One 5: e8625.

25. Wang S, Diller KR, Aggarwal SJ (2003) Kinetics study of endogenous heat shock protein 70 expression. J Biomech Eng 125: 794-797.

26. Yamasaki A, Takahashi T, Suzuki T, Fujiwara T, Hirakawa M, et al. (2001) Differential effects of isoflurane and halothane on the induction of heat shock proteins. Biochem Pharmacol 62: 375-382.