Original Contribution

A Benzylidene Lactam, KNK437 Inhibits the Expression of Heat Shock Proteins in Human Oral Cancer Cell Lines Exposed to Hyperthermia

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Abstract : Single exposure of cells to hyperthermic treatment, makes them thermotolerant to subsequent heat treatment. Tumor cells that express normal or elevated levels of HSPs show thermotolerance. KNK437, a chemically synthesized benzylidene lactam compound, has been specifically found to inhibit the synthesis of heat shock proteins and thus leading to significant reduction in the thermotolerance of cells. However, mechanisms by which KNK437 inhibits thermotolerance are still not clearly understood. In this study an attempt has been made to elucidate the mechanisms of inhibition of HSPs by KNK437 in 2 cell lines of human oral squamous cell carcinoma with varying sensitivities (HSC4 ; heat-resistant and KB ; heat-sensitive) with an aim to identify which of the HSPs are targeted by KNK437. Single administration of 100 μ M KNK437-treatment always resulted in a higher than 85% cell survival compared to the untreated groups, indicating the KNK437 had a minimum cytotoxic effect in both cell lines. Combination of hyperthermia with KNK437 after heat shock, specifically inhibited the induction of HSPs, where the expression of HSP27, HSP40, HSP70 and HSP90 reduced in a time dependent manner. Interestingly, the hyperthermic treatment combined with KNK437 produced supra-reductive effect on the expression of HSP27 and HSP90 in HSC4, HSP27, HSP40 and HSP90 in KB cells, when compared to heat treatment alone. Our results indicate that inhibition of HSPs by KNK437 plays an important role in inhibiting the thermotolerance of the cells and may be a valuable to improve the efficacy of oral cancer during hyperthermic therapy.

Key Words : HSP, hyperthermia, KNK437, oral cancer.

Introduction

In recent years, hyperthermia is performed as one of the multidisciplinary approach to treat cancer.

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Induction of HSPs during the hyperthermic treatment of cancer is a major hurdle to obtain better therapeutic gains. Therefore, a specific inhibitor of HSPs induction may be helpful to improve the effectiveness of heat treatment in cancer therapy. Exposure of cells to heat shock, results in synthesis of heat shock proteins (HSPs)¹⁾ leading to thermotolerance in the tumors²⁾.

HSPs are a large family of conserved proteins classified as high-molecular-weight HSPs, such as HSP70, HSP90, and HSP110, or low-molecular-weight HSPs, which have molecular masses ranging between 10 to 30³⁻⁵⁾. HSPs function as chaperone proteins⁶⁾, and play an important cellular protective role against a variety of stresses⁷⁻⁹⁾. Up-regulation of HSPs has been reported in several cancers, including breast, kidney, and bladder cancers and leukemia⁶⁾ and thought to play a role during cell proliferation¹⁰⁾ since HSPs are essential for survival of cancer cells in various types of cancers⁶⁾. It is known that HSP70 and HSP27 are the most stress-inducible members of the HSP families, whereas HSP90 is constitutively expressed and less stress inducible. HSP70 and HSP27 have been suggested to interfere with transduction of apoptotic signal⁶⁾⁽¹¹⁾⁽¹²⁾. HSP40 chaperones are involved in nearly all aspects of protein synthesis and secretion because of their importance in HSP70 function. They are also thought to have an intrinsic ability to bind and fold some misfolded proteins¹³⁾. HSP90 is a molecular chaperone that is involved in folding, activation and assembly of several proteins, known as HSP90 client proteins¹⁴⁾⁽¹⁵⁾. Recently, inhibition of HSPs has been developed as a unique therapeutic target in cancer therapy because a large number of oncogenic client proteins or pathways are involved¹⁶⁾. Shin *et al.* found that KRIBB3 (5-(5-ethyl-2-hydroxy-4-methoxyphenyl)-4-(4-methoxyphenyl) isoxazole) bound to HSP27 and inhibited cell migration by blocking PKC-dependent HSP27 phosphorylation¹⁷⁾. Naturally occurring compounds, such as geldanamycin and radicicol, have been known to inhibit HSP90 action¹²⁾.

KNK437, a benzylidene lactam compound synthesized by Kaneka Corporation (Osaka, Japan), has been reported to inhibit the synthesis of HSPs and the acquisition of thermotolerance at mRNA level. However, the precise mechanisms of inhibition of HSPs by KNK437 have yet been elucidated¹⁸⁾. Furthermore, KNK437 has been found to be non-toxic to the cells. It would be a major step forward if the mechanisms of inhibition by KNK437 could be understood for the efficient application of hyperthermia or HSP-targeted therapy in cancers. In the present study an attempt has been made to unfurl the mechanisms of inhibition of HSPs by KNK437 in two oral cancer cell lines with a specific aim to identify target HSPs that are targeted by KNK437.

Materials and Methods

Cell lines and culture

Human oral cancer cell lines (HSC4 and KB) were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, Japan. The cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical Company, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Utah, USA), 100 μ g/ml penicillin (Meiji Seika, Tokyo, Japan) and 100 μ g/ml streptomycin (Meiji Seika, Japan) in a CO₂ incubator (Sanyo Electric Company, Osaka, Japan) in an atmosphere of 5% CO₂ in 95% air at 37°C. Exponentially growing cells (5 $\times$ 10⁵) were seeded into 25 cm² flasks (Sumitomo Bakelite Co. Ltd., Tokyo, Japan) 24 h before each treatment.

Chemicals

KNK437 (*N*-formyl-3, 4-methylenedioxy-benzylidene- γ -butyrolactam) (Kaneka Corp., Osaka, Japan) was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) and added into culture medium to the final concentration indicated for each experiment.

Heat treatment

24 h after seeding, the cells (HSC4 and KB) were heated by totally immersing the flasks in a temperature controlled circulating water bath (TAITEC Co., Ltd., Saitama, Japan) at 45°C for 0 to 100 min duration. The temperature was controlled within $\pm 0.05^\circ\text{C}$.

KNK437 treatment

The cells were treated with 0, 50, 100, 150, 200, 300 μM KNK437 alone by replacing the growth medium with the medium containing the above concentrations of KNK437 and then incubating the cells for 6 h. Immediately after the elapse of 6h the cells were rinsed three times with KNK437-free medium and their clonogenicity was determined.

Heat and KNK437 treatment

The cells were exposed to 100 μM KNK437 containing medium at 37°C for 6 h after heating at 45°C.

Cell survival assay

The modulation of heat induced cytotoxicity by KNK437 was estimated using a colony-forming assay¹⁹⁾. Cells were trypsinized, counted, and replated at the appropriate dilutions. The cells were allowed to grow for 10-14 days at 37°C in a CO₂ incubator. The resultant colonies were fixed in 10% formaldehyde and stained with 10% Giemsa and clusters containing 50 or more cells were scored as a colony. The plating efficiency of cells was determined and the surviving fraction was fitted on a linear quadratic equation $\text{SF} = \exp^{-(\alpha D + \beta D^2)}$.

Western blotting

Protein in cell free extracts were separated by electrophoresis in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). The individual proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA) using an electrophoretic transfer apparatus (TEFCO Co. Ltd., Tokyo, Japan) at room temperature for 1 h. The blotted membranes were blocked for 1 h in TBS-T (containing 0.1% Tween 20) plus 5% powdered skimmed milk, and conjugated for 1 h with anti-HSP27 monoclonal antibody (Ab-1, Oncogene, Science, Inc., Uniondale, NY, USA) diluted 1 : 1600 in TBS-T, anti-HSP40 polyclonal antibody (C-20, Santa Cruz Biotechnology, Inc., CA, USA) diluted 1 : 800 in TBS-T, mouse anti-HSP70 monoclonal antibody (Stressgen, Victoria, Canada) diluted 1 : 1000 in TBS-T, anti-HSP90 monoclonal antibody (Calbiochem, San Diego, CA, USA) diluted 1 : 1000 in TBS-T. The membranes were then washed three times in TBS buffer, and incubated for 1 h with an appropriate secondary antibody horseradish peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) (ImmunoResearch Laboratories Inc., West Grove, PA) diluted

1 : 5000 in TBS-T for HSP27, HSP70, HSP90, and Anti-Goat Immunoglobulins HRP (DakoCytomation Denmark) diluted 1 : 5000 in TBS-T for HSP40. Conjugation of antibody was detected using ECL plus kit (Amersham Pharmacia Biotech Inc., Little Chalfont, UK) according to the manufacturer's instructions. To quantify the amount of protein loaded per lane, Western blotting for beta-actin was used as control. The mouse monoclonal antibody for beta-actin (beta-actin AC-15-ab6276, Abcam, Cambridge, UK) was diluted to 1 : 25000 in TBS-T and incubated for 1 h with an appropriate secondary antibody and horseradish peroxidase-conjugated AffiniPure goat anti-mouse IgG (H+L) (Immuno Research Laboratories, West Grove, PA, USA) in TBS-T and utilized in the same manner. The intensities of the bands were quantified using Image J 1.33 u (National Institutes of Health, USA).

Statistical analysis

Statistical analysis was conducted with JMP 5.1.1 J software (SAS Institute Inc. Cary, NC). The

Fig. 1. Effect of heat treatment on the clonogenicity of HSC4 and KB cells and HSP protein expressions. (a) Clonogenicity : Cells were exposed to heat at 45°C for various times ranging from 0 to 100 min. KB cells are more sensitive to heating than HSC4 (LD₁₀ at 45°C 40 min for HSC4 ; 17min for KB). Symbols : HSC4 △ ; KB □. Standard errors are shown for each concentration. (b) Western blot analysis of the HSPs in oral cancer cell lines. (c) Intensities of the bands were quantified by the proportion of HSPs versus beta actin with Image J 1.33 u (National Institutes of Health, USA). The relative band intensity represents the intensity of KB group / HSC4 group. The significance of the differences was tested by the Student's *t*-test : *p* < 0.05. # *p* < 0.05 compared with HSC4 group. Each column is the average of three measurements ; bars, S.D.

Student's t-test was used for comparisons between the values and a $p < 0.05$ was considered statistically significant.

Results

Cellular sensitivity to heating and HSP protein expressions

Thermosensitivity of HSC4 and KB cells was determined by heating these cells for 0-100 min at 45°C. Heating of HSC4 and KB cells for different times showed a time dependent decline in the clonogenicity of cells (Fig. 1). A maximum decline in the cell survival was observed at 100 min for HSC4 cells whereas 50 min for KB cells. The LD₁₀ at 45°C was found to be 40 min for HSC4 and 17 min for KB cells respectively. The molecular mechanisms of differential sensitivity of HSC4 and KB cells were determined by HSP expression after heating, since the amount of HSPs under non-stressed condition is regarded as a barometer of heat sensitivity of cells. The results of our study have shown that levels of HSP27 and HSP40 were lower in KB cells indicating higher thermosensitivity than HSC4, whereas the expression of HSP70 was higher in KB than HSC4 cells (Fig. 1b, c).

Cellular sensitivity with KNK437

The effect of various concentrations of KNK437 was studied in HSC4 and KB cells. Treatment of HSC4 and KB cells with varying concentration of KNK437 showed a concentration dependent decline in the clonogenicity of both cells (Fig. 2). However, the differences between various concentrations of KNK437 were statistically non-significant. The surviving fraction was always greater than 85% in both the cell lines treated with 100 μ M of KNK437 indicating that this concentration of KNK437 had a minimum toxicity in both HSC4 and KB cells (Fig. 2). The highest concentration of 300 μ M of KNK437 also showed a low toxicity as evidenced by a marginal decline in the clonogenicity of cells when compared with 100 μ M KNK437.

The effect of heating or combined with KNK437 on HSP expressions

Effect of heating was examined on the synthesis of HSPs in HSC4 and KB cells exposed to LD₁₀ heat treatment at 45°C. Heat treatment increased the expression of HSP27, HSP40, HSP70, and HSP90 in a time dependent manner in both cell lines (Fig. 3a). It is interesting to note that the expression of HSPs after heat treatment was more pronounced in KB cells (*i.e.* heat-sensitive) than in the HSC4 cells (*i.e.* heat-resistance). Especially, the expression of HSP40 was

Fig. 2. Effect of KNK437 on the cell survival. Cells were treated with a single treatment of various concentration of KNK437 and cell survival evaluated by colony forming assay. The results show that following treatment of both cells with varying concentrations of KNK437 there is a marginal decline in the surviving fraction and even 300 μ M KNK437 did not show high cytotoxicity. Symbols: HSC4 Δ ; KB $\square$. Standard errors are shown for each concentration.

Fig. 3. The effect of heating on HSP expressions. (a) Western blot analysis of the HSP protein after hyperthermia at LD₁₀ at 45°C. (b) Image J 1.33 u analysis of western blotting results. The relative band intensity represents the intensity of 4 h or 8 h groups / 0 h group. In both cell lines, HSP27, HSP40, HSP70, and HSP90 were induced after heating in a time dependent manner. The significance of the differences was tested by the Student's *t*-test: $p < 0.05$. # $p < 0.05$ compared with 0 h group. Each column is the average of three measurements; bars, S.D.

higher in the KB cells after heat treatment when compared to the cells that did not receive heat treatment. The expression of various HSPs depended on the length of heat treatment. The maximum expression of all HSPs was observed at 8 h in both HSC4 and KB cells. HSP40 was highly expressed in both HSC4 and KB cells when compared to the other HSPs. The degree of expression of various heat shock proteins in HSPs was lesser in HSC4 cells when compared with the KB cells where all heat shock proteins were expressed relatively more (Fig. 3b).

In another experiment an attempt has been made to estimate the expression of various heat shock proteins after heat and KNK437 treatment. Treatment of HSC4 and KB cells with 100 μ M KNK437 significantly reduced the expression of HSP27, HSP40, HSP70 and HSP90 proteins. This reduction in HSC4 expression was greater especially HSP27 and HSP40 in KB than HSC4 cells (Fig. 4b). Furthermore, this decline in HSPs was effected earlier in KB cells than the HSC4 cells. The inhibition of HSP27 expression was greater after heat and KNK437 treatment in both cell lines when compared to heat treatment alone (Fig. 3 and 4). It seems that combined treatment of heat and KNK437 produced supra-reductive effects (*i.e.* HSP27 and HSP90 in HSC4, HSP27, HSP40 and HSP90 in KB) compared to the KNK437-untreated group.

Fig. 4. The effect of heating combined with KNK437 on HSP expressions. (a) Western blotting results. Cells were treated LD₁₀ heating at 45°C and then, with 100 μ M KNK437 (b) Image J 1.33 u analysis of western blotting results. The relative band intensity represents the intensity of 4 h or 8 h groups / 0 h group. The significance of the differences was tested by the Student's *t*-test: $p < 0.05$. # $p < 0.05$ compared with 0 h group. Each column is the average of three measurements; bars, S.D. Both cell lines inhibited the induction of HSP expressions in time dependence.

Discussion

Thermotolerance is a major obstacle in using hyperthermia as an effective treatment modality. Thermotolerance is a transient heat induced resistance by prior hyperthermia²⁰⁾²¹⁾. Inhibition of thermotolerance may be helpful to improve the therapeutic outcome during heat treatment. Thermosensitivity and cytotoxic effect of hyperthermia can be increased by inhibiting expression of various HSPs to the clear advantage of patients undergoing thermotherapy of tumors. It is important to examine thermosensitivity by the expression of various HSPs. The expression of various HSPs had difference in the HSC4 cells and KB cells (Fig. 1). However, in this study result, it is difficult to prove the relationship between the HSPs expression and thermosensitivity. With the cell lines which various kinds of thermosensitivity is different in, it is necessary to examine the relationship between the HSPs expression and thermosensitivity. The present study was designed to understand the mechanisms of increased theramosensitivity by KNK437 in HSC4 and KB cells. KNK437 does not show any anticancer effect by itself, nor does it inhibit the activities of protein kinases A and C and tyrosine kinase, indicating that drug itself does not interfere with the mechanisms responsible for the cytotoxic effect of other

neoplastic agents. This makes it a good candidate that might be a more useful potentiator of hyperthermic treatment. Under our experimental condition, single treatment of HSC4 and KB cell with 100 μ M KNK437 did not cause appreciable cytotoxic effect as the survival of KNK437 treated cells was always higher than 85% compared to the untreated controls. Even the highest concentration of KNK437 (300 μ M), had low toxic effect on the cell survival.

Thermosensitivity of HSC4 and KB was determined by heating these cells for 0-100 min at 45°C (Fig. 1). Heating of HSC4 and KB cell showed a time dependent decline in the clonogenicity of cells. A similar effect has been observed earlier in human carcinoma cells¹⁸⁾. Interestingly, in this study, the combined treatment with heating and KNK437 produced supra-reductive effects on HSPs (*i.e.* HSP27 and HSP90 in HSC4, HSP27, HSP40 and HSP90 in KB) compared to the result obtained with heat treatment alone owing to their important role in signaling proteins such as steroid receptors, Raf, serine kinases, cyclin-dependent kinase 4 (CDK-4) and some receptor kinases that are essential for cell function. KNK437 seems to target both HSP27 and HSP90 in both oral cancer cell lines. In recent years, inhibition of HSP90 has been developed as a unique therapeutic target for cancer treatment as disruption, because a large number of its function leads to the depletion of multiple oncogenic client proteins or pathways¹⁶⁾²⁰⁻²³⁾. A new derivative of geldanamycin (17-AAG) also inhibits the chaperone function of HSP90 in a similar manner²⁴⁾ but shows less toxicity²²⁾. This drug has been shown to possess antitumor activity in a number of cell lines and several human xenograft models, including colon, breast, and prostate cancer¹²⁾²³⁾. Recently, the histone deacetylase inhibitors, one of the novel anticancer agents, have also been shown to induce acetylation of HSP90 and inhibit its chaperone function²⁴⁾²⁵⁾.

In addition to the obvious degradation of HSP90 and HSP27 by combined treatment with heat and KNK437, the inhibition of the heat-inducible HSP70 was also observed. This is consistent with the previous report by Yokota *et al.* where KNK437 has been found to inhibit the heat-inducible HSP70 and the acquisition of thermotolerance in COLO 320DM cells¹⁸⁾. However, in our study a complete inhibition of HSP70 was not discernible as there was a low level of HSP70 expression, in both cells (Fig. 4). This might be due to the fact that HSP70 is a co-chaperone of HSP90 and is induced when HSP90 is inhibited²⁶⁾. Many previous reports have pointed that HSP90 inhibitors also function as HSP inducers¹⁰⁾¹²⁾²⁶⁾²⁷⁾ previously reported that HSP90 inhibitor geldanamycine could induce the expression of HSP70 and HSP27 probably because of it induced proteotoxic stresses via inhibition of HSP90. Therefore, the abrogation of HSP70 induction would further increase the efficacy of HSP90 inhibitors. Quercetin, a bioflavonoid, was previously reported to inhibit the induction of HSP70 synthesis after heat shock and induce apoptosis in tumor cells²⁸⁾. Yokota *et al.* reported that KNK437 is more effective than quercetin in inhibiting the induction of HSPs and inhibiting the acquisition of thermotolerance¹⁸⁾. Guo *et al.* reported that cotreatment with KNK437 and 17-AAG attenuated 17-AAG-mediated HSP70 induction and increased 17-AAG-induced apoptosis and loss of clonogenic survival of HL-60 cells compared with the treatment with either agent alone¹²⁾. Furthermore, it is reported that KNK437 has inhibiting effects on histone H3 lysine 4 methylation²⁹⁾, as a result, possibility to restrain genes expression of HSPs is suggested by heating combined with KNK437. However, it is necessary to make clear that the expression of HSP27, HSP40 and HSP90 in KB cells decreased by heating combined with KNK437.

The precise mechanisms of the inhibition of KNK437 remains to be elucidated, however, it is clear

from our study that KNK437 inhibited the expression of HSPs and therefore it may be a suitable candidate to target HSPs and improve the treatment of cancer by hyperthermia in clinical situation.

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口腔癌細胞株における温熱処理後の 熱ショック蛋白質誘導への KNK437 の影響

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要 旨：KNK437 は、特異的に HSP の誘導を阻害し、温熱耐性を抑制する可能性があるが、その作用機序は未だ明確ではない。われわれは、温熱感受性の異なるヒト口腔内悪性腫瘍由来細胞株を用い、KNK437 が HSP に与える影響を分析した。用いた細胞株において、KNK437 (100) の細胞毒性はわずかであった。温熱処理後に KNK437 を投与することにより、HSP27、HSP40、HSP70 および HSP90 の誘発を経時的に抑制した。興味深いことに、HSP の誘発量は、KNK437 を投与すると、KNK437 非投与群より減少した。HSP の誘発を抑制する KNK437 の作用は、口腔内悪性腫瘍に対する温熱療法の有効性を改善する価値を示唆した。