The Effects of KNK437, a Novel Inhibitor of Heat Shock Protein Synthesis, on the Acquisition of Thermotolerance in a Murine Transplantable Tumor in Vivo

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ABSTRACT

A newly synthesized reagent, KNK437, has been found specifically to inhibit the synthesis of heat shock proteins in vitro. In this study, we investigated the effects of KNK437 on the synthesis of heat shock proteins and the induction of thermotolerance in transplantable tumors in vivo. SCC VII cells were grown in vivo and transplanted into C3H/He mice. The concentrations of KNK437 in the tumors and the sera of the mice were examined by high-performance liquid chromatography. Hsp72 synthesis was examined by Western immunoblot analysis. The response to hyperthermia was evaluated in terms of the delay in tumor growth. KNK437 had low toxicity in vivo. The concentration of KNK437 in the tumors gradually increased and reached a peak 6 h after i.p. injection. Hsp72 were synthesized 8 h after hyperthermia at 44°C for 10 min, and their synthesis was inhibited by administration of KNK437 6 h before hyperthermia. At a concentration of 200 mg/kg, KNK437 alone showed no antitumor effects and did not increase the thermosensitivity of nontolerant tumors. The same dose of KNK437 enhanced the antitumor effects of fractionated heat treatment at 44°C in a synergistic manner. This study strongly suggests the inhibition of thermotolerance via the inhibition of HSP72 in vivo. The inhibition of thermotolerance by KNK437 may help to improve the efficacy of clinical fractionated hyperthermia.

INTRODUCTION

Hyperthermia has been studied biologically (1) and clinically (2) as a modality of cancer therapy. However, there are still several serious clinical problems to be resolved. One major problem is the induction of thermotolerance in the tumor (3). Thermotolerance is the increase in survival of cells at elevated temperatures after heat pretreatment. Materials that inhibit the induction of thermotolerance may help to improve the effects of fractionated hyperthermia.

When cells or organisms are exposed to heat shock, they respond by synthesizing a group of proteins called HSPs (1). In other studies, we have found that quercetin, a bioflavonoid, specifically inhibits both the synthesis of HSPs and the acquisition of thermotolerance (4, 5).

Recently, KNK437, a newly synthesized reagent produced by Kaneka Corporation (Takasago, Japan), was found to inhibit the synthesis of HSPs and the acquisition of thermotolerance in vivo (6). In this study, we investigated the effects of KNK437 on the induction of heat shock proteins and the acquisition of thermotolerance in vivo.

MATERIALS AND METHODS

Animal Tumor Production. Male C3H/He mice 10 weeks of age (obtained from Shimizu Jikken Dobutsu, Kyoto, Japan) were used. They were caged in groups of eight at a constant temperature and with free access to food and drinking water. The transplantable murine tumors were SCC VII carcinoma cells. The foot of the right hind limb of each mouse was inoculated s.c. with 0.5–1 × 105 viable tumor cells, and the resultant tumors were used for treatment 14 days later when the mean tumor diameter had reached 10 mm.

Hyperthermia. To subject the tumors to hyperthermia, the right foot of each animal was immersed in a water bath (KT35D; ADVANTEC, Tokyo, Japan) maintained at the desired temperature to an accuracy of ± 0.05°C. After the mouse had been anesthetized with 50 mg/kg pentobarbital sodium solution (Abbott Laboratories, North Chicago, IL), the tumor-bearing leg was pulled down into the water bath using a special sinker (weighing ~45 g) taped to the skin of the toe. Care was taken not to impair the blood flow in the limb. While the extended right leg was receiving local heat, the mouse was air-cooled. All temperatures mentioned in this paper are those of the water bath.

The intratumor thermometry data have been described previously (7). Briefly, the temperature at the tumor center reached equilibrium within 1–2 min after the leg had been immersed in...
the water bath; it remained 0.2–0.3°C below the water-bath temperature.

**Treatment with KNK437.** KNK437 (Fig. 1a), a newly synthesized reagent produced by Kaneka Corporation (6) as N-formyl-3,4-methylenedioxy-benzylidene-γ-butyrolactam, was dissolved in olive oil immediately before use. The KNK437 was administered i.p. 6 h before the first heat treatment. It was used mainly at a concentration of 200 mg/kg. The metabolized form of KNK437 is KNK423 (Fig. 1b). The effect of both reagents in terms of the inhibition of the synthesis of HSPs and the acquisition of thermotolerance in *vitro* is the same (6). The concentration of these chemicals in both the tumors and the sera was measured by HPLC.

**HPLC Analysis of KNK437 and an Effective Metabolite (KNK423).** Animal tumor samples (0.2 g) were minced and homogenized in 5 ml of ethyl acetate. The homogenate was centrifuged at 2000 × g for 5 min. The supernatants were evaporated to dryness, and then the residues were reconstituted in 200 μl of methanol. Aliquot (40-μl) samples were injected into the HPLC system.

Serum samples (0.2 ml) were poured on to Bond Elut C-18 cartridges conditioned with 3 ml of acetonitrile, 3 ml of water, and 3 ml of 2% (v/v) phosphoric acid. The cartridges were washed with 3 ml of water and dried by air. The eluates obtained with 3 ml of acetonitrile were collected into glass tubes and evaporated to dryness; the residues were reconstituted in 200 μl of methanol. Aliquot (40-μl) samples were injected into the HPLC system.

The analytical column was a J'sphere ODS-H80, 4 μm, 4.6 × 250 mm (YMC, Kyoto, Japan), and the guard column was a Sumipax Filter PG-ODS (Sumika Chemical Analysis Service, Osaka, Japan). Each was used at a column temperature of 40°C. The isocratic mobile phase was acetonitrile:methanol:water (23:20:57; v/v). The flow rate was 0.5 ml/min, and peaks were detected at wavelengths of 346 nm (KNK437) and 319 nm (KNK423).

**SDS-PAGE and Immunoblot Analysis with Anti-Hsp72 Antibody.** Immediately after treatment, the tumors were totally excised and frozen. The frozen samples were crushed with a wood hammer on a cooled stainless-steel plate. The weight of each sample (50–100 mg) was measured in a microtube. Laemmli’s SDS sample buffer with DTT and without bromphenol blue was added to 10× the volume of the sample; this mixture was then agitated vigorously in a microtube and then sonicated. It was boiled for 5 min, then centrifuged at 14,000 rpm for 5 min. The protein in the supernatant was weighed (Bio-Rad Laboratories, Hercules, CA) and then prepared by SDS sample buffer with DTT and bromphenol blue. The solution was then boiled for 5 min and stored at −20°C.

One-dimensional 10% SDS-PAGE was performed according to the method of Laemmli (8). The samples were loaded with 30 μg/lane. Electrophoresis was done using minigels at 25 mA/gel for 70 min. SDS-polyacrylamide slab gels were transferred on to polyvinylidene fluoride membranes at 2 mA/cm² by a semidy blotter. Blocking incubation was performed in a 5% skim milk solution (Bio-Rad Laboratories) rotating for 1 h at room temperature and then stored at 4°C overnight. Blotted filters were incubated with the primary murine monoclonal antibody against human HSP72 (C92F3A-5; StressGen Biotechnologies Corporation, Victoria, British Columbia, Canada) diluted 500-1000× with 5% skim milk, sealed in a vinyl pack, and rotated for 90 min at room temperature. Hors eradish peroxidase-antimouse IgG (Bio-Rad Laboratories), diluted 2000–5000× with 5% skim milk, was used as the secondary antibody; the solution was sealed in a vinyl pack and rotated for 70 min at room temperature. It was followed by detection with enhanced chemiluminescence Western blotting detection reagents (Amer sham, Little Chalfont, England). The blot was exposed to X-ray film (Fuji RXO-H film; Fuji Photo Film Co. Ltd, Kanagawa, Japan).

**Growth Delay Assay.** The tumor response to heat was evaluated in terms of the delay in tumor growth. A caliper was used to measure the three dimensions of each tumor every 2 days after the treatment. The number of days it took for a tumor to triple in volume was used as a measure of the antitumor effect of the treatment. Each treatment group consisted of at least 19 animals. The data points shown in this study represent mean values, and the time for tumor volume to triple (Table 1) was measured from each tumor growth curve. Statistically significant differences were determined using the *t* test and the *χ*² test.

**RESULTS**

KNK437 is a weakly toxic drug. We wanted to investigate whether a nontoxic dose could enhance the antitumor effect of...
fractionated hyperthermia. To determine the toxicity of KNK437, we studied the changes in body weight of tumor-free CD-1 (ICR) mice (obtained from Charles River Japan, Yokohama, Japan) that had been given various doses (Fig. 2). The KNK437 was administered i.p. on the first day at concentrations ranging from 62.5 to 500 mg/kg. At KNK437 concentrations of up to 400 mg/kg, body-weight losses were recovered by 2 days after treatment.

The concentrations of KNK437 and KNK423, an effective metabolized form of KNK437, in the tumor and the sera were examined by HPLC (Fig. 3a and b). KNK423 was detected at much higher levels than KNK437 in both the sera and the tumors at all periods. Higher levels of KNK423 were observed in the sera than in the tumor after i.p. administration. These levels averaged 83.26 μg/ml and 21.46 μg/g at the peak point, respectively. There was a greater drug accumulation of KNK423 in the tumor after i.p. injection than after p.o. feeding. 21.46 and 5.34 μg/g at the peak point, respectively. The peak concentration of KNK423 in the tumor occurred 6 h after injection.

KNK437 alone did not show any antitumor effect in terms of tumor growth delay (Fig. 4). Control tumors tripled their growth in 10.5 days, and tumors treated with KNK437 at a concentration of 200 mg/kg tripled their growth in 9.85 days, indicating no drug effect on tumor growth.

We then used anti-Hsp72 antibody to examine the synthesis of Hsp72 in the SCC VII tumors by Western immunoblot analysis (Fig. 5). The synthesis of Hsp72 in the SCC VII tumors was inherently weak, and the induction of Hsp72 by heat shock (as measured in tumors resected either immediately or 2 h after heating) was not significant. The HSP72 band thereafter became denser until the end of the examination, 8 h after heating. KNK437 administered 6 h before heating inhibited the induction of Hsp72 (Fig. 5). The HSP72 band had become slight weaker 24 h after heating.

Treatment with KNK437 did not affect the growth of tumors after a single heat treatment at 44°C for 30 min. The times required for single-heated tumors with and without KNK437 to triple in size (mean, 14.15 and 14.02 days, respectively) were not statistically different (Fig. 6; Table 1).

To induce thermotolerance, we treated the SCC VII tumors at 44°C for 10 min and then placed the tumor-bearing mice in their cages at room temperature for 8 h to allow them to recover. The acquisition of thermotolerance was demonstrated by the growth delay of the tumors (Fig. 6). As shown by t test, there was a statistically significant reduction (mean, 3.5 days) in the time taken for tumors that had been subjected to repeated heat shock (44°C for 30 min) to triple in volume, in comparison with nontolerant tumors (Fig. 6). This indicated that the preheat-shocked tumors had gained heat resistance.

KNK437 inhibited the induction of thermotolerance at a concentration of 200 mg/kg when it was administered i.p. 6 h before the initial heating (Fig. 6). After the tumors treated with KNK437 had been heated at 44°C for 30 min, the time required for them to triple in volume was an average of 2.9 days longer than that for tumors that had been heat-shocked without KNK437 treatment. The result was statistically significant according to t test ($P < 0.05$). These results were duplicated.

**DISCUSSION**

Little is known about the newly synthesized reagent KNK437 (Fig. 1a). We know that it specifically inhibits the synthesis of HSPs and the acquisition of thermotolerance *in vitro* (6). It has also been found that KNK437 inhibition was recognized at the mRNA level, and this has been confirmed by Northern blot analysis (6).

We used Western immunoblot analysis to examine the synthesis of Hsp72 in the SCC VII tumors (Fig. 5). The induction of Hsp72 by heating at 44°C for 10 min, as measured in tumors resected immediately or 2 h after heating, was minimal, but it became prominent 8 h after heating. Decay in Hsp72 levels was observed 24 h after heating. KNK437 inhibited the induction of Hsp72 when it was administered 6 h before heating.
Heat sensitization by KNK437 was not observed in this study (Fig. 6). After KNK437 administration, heating to 44°C for 30 min did not make the tumors heat sensitive. Exposure to this temperature might have caused severe cellular, vascular, and histological damage. This kind of exposure would have exceeded the protective capacity of the HSPs, so that inhibition of the synthesis of HSPs would not have been critical for cell killing. The lack of heat sensitization by KNK437 was expected based on the in vitro data. There is no effect on survival by single heat at 42°C for 90 min or at 44°C for 10 min with or without KNK437 in vitro (6). It is not clear whether the same result would be drawn for longer heating. At least, there is no effect on survival by single heat at 44°C for 10 min with or without HSP induction. The results of the in vivo studies were comparable with those of the in vitro ones (6).

Hyperthermia to 44°C for 10 min had little effect on tumor growth but was sufficient to lead to thermotolerance in vivo (Fig. 6). KNK437 inhibited the acquisition of thermotolerance (Fig. 6; Table 1). The results indicate that the effect of KNK437 on the induction of thermotolerance contributed to the inhibition of the synthesis of HSPs not only in vitro but also in vivo.

A bioflavonoid, quercetin, is known to inhibit the synthesis of stress proteins. The inhibitory mechanism of the stress response in heat-shocked cells has been investigated extensively. Although quercetin has been reported to inhibit the induction of HSP70 synthesis at the mRNA level (4), the inhibitory mechanism has not been clearly established. The binding of HSF1 to the heat shock element is blocked when quercetin is added before and during heat treatment (9), and the phosphorylation of HSF1 is partly inhibited (10). Nagai et al. (10) have reported that the decline of the HSF1-heat shock element complex might be linked to the decrease of HSF1 levels caused by quercetin. This is consistent with a recent report that the targeted disruption of murine HSF1 abolishes thermotolerance as well as protection against heat-inducible apoptosis (11). On the other hand, quercetin is also reported only to delay the synthesis of HSP70 after heat shock (12, 13). Like quercetin, KNK437 inhibited the induction of HSP72 at the mRNA level (6), and this inhibitory mechanism is now being investigated.

The mechanism of thermotolerance development, either in vitro or in vivo, is still unresolved. At the cellular and molecular level, several pathways of thermotolerance induction have been
proposed (14–18). The development of thermotolerance has been reported frequently both in vivo and in vitro but in relation to HSP72 (19, 20). A correlation between hsp40 synthesis and the degree of thermotolerance has been demonstrated in murine squamous cell carcinoma in vivo (20). Our study has shown a relationship between the development of thermotolerance and HSP72 synthesis in vivo through the inhibitory effect of KNK437. KNK437 may contribute to improving the effects of clinical fractionated hyperthermia.

REFERENCES


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