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The effect of KNK437, a heat shock protein inhibitor, on angiogenesis of endothelial cells

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Aim: Hyperthermia is an effective therapy for cancer and tumor angiogenesis. However, the development of thermotolerance is an important problem. We aimed to investigate the effects of KNK437 and quercetin on angiogenesis and thermotolerance in endothelial cells (ECs).

Materials and methods: ECs were grown in EGM-2 medium. ECs were preincubated with KNK437 or quercetin and then treated with single and fractionated hyperthermia. Tube formation assay on Matrigel Matrix was performed in these cells to investigate angiogenesis.

Results: Tube formation was suppressed in cells that were treated with KNK437 and quercetin at 37 °C. Single hyperthermia (45 °C for 1 h) inhibited tube formation. KNK437 and quercetin pretreatment decreased capillary-like structures more effectively than single hyperthermia treatment. In cells that acquired thermotolerance induced by fractionated hyperthermia, tube formation was dramatically increased, but KNK437 and quercetin reversed the effects of thermotolerance on angiogenesis. KNK437 was found to be more effective than quercetin in all study groups.

Conclusion: This study is original in that KNK437 inhibited angiogenesis and thermotolerance in ECs. This compound may be an essential molecule contributing to the efficient treatment of hyperthermic therapy in human cancers.

Key words: Hyperthermia, angiogenesis, KNK437, quercetin, tube formation, endothelial cells

Isı şok protein inhibitörü KNK437'nin endotel hücrelerinde anjiyogenez üzerine etkisi

Amaç: Hipertermi, kanser ve tümöral anjiyogenez için etkili bir tedavi şeklidir. Ancak, termotolerans gelişimi önemli bir problemdir. Bu çalışmada KNK437 ve quercetinin endotel hücrelerinde anjiyogenez ve termotolerans üzerine etkileri araştırıldı.

Yöntem ve gereç: KNK437 veya quercetin ile ön muamele edilmiş olan endotel hücrelerine tek basamaklı ve kademeli hipertermi uygulanmıştır. Bu hücrelerde anjiyogenezin incelenmesi için Matrijel Matrikste tüp oluşumu deneyi gerçekleştirilmiştir.

Bulgular: KNK437 veya quercetin ile 37 °C'de muamele edilmiş olan hücrelerde tüp oluşumunun baskılandığı görülmüştür. Tek basamaklı hiperterminin (45 °C'de 1 saat) tüp oluşumunu azalttığı görülmüştür. Tüp oluşumu, KNK437 veya quercetin ile ön muamele edilmiş hipertermi grubunda, sadece hipertermi uygulanmış gruba göre düşük bulunmuştur. Kademeli hipertermi grubuna ait tüp uzunluğu anlamlı şekilde yeniden artış göstermiştir. Bununla birlikte, KNK437 veya quercetin ile ön muamele termotoleransın anjiyogenez üzerine olan etkilerini geriye döndürmüştür. Çalışılan tüm deney gruplarında KNK437'nin quercetinden daha etkili olduğu görülmüştür.

Sonuç: Bu çalışma, KNK437'nin endotel hücrelerinde anjiyogenez ve termotoleransı inhibe etmesi bakımından orjinaldir. KNK437 insan metastatik kanserlerinde uygulanan hipertermik tedavinin etkinliğine katkıda bulunabilen esansiyel bir molekül olabilir.

Anahtar sözcükler: Hipertermi, anjiyogenez, KNK437, quercetin, tüp oluşumu, endotel hücreleri

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Introduction

Angiogenesis, new blood vessel formation, is essential for tumor progression. The use of angiogenesis inhibitors is known to be a useful approach to fighting cancer (1,2).

Hyperthermia is known to be an ideal combination therapy for tumors due to its slight side effects and low toxicity (3). This therapy has been shown to have a synergistic effect with traditional cancer therapies. In vitro and animal hyperthermic experiments exhibited a direct cell killing effect at temperatures ranging from 41 to 47 °C (4,5).

One major problem of hyperthermia treatment is the induction of thermotolerance in the tumor (6). Materials that inhibit the induction of thermotolerance may help to improve the effects of fractionated hyperthermia. Quercetin (3,3',4',5,7-pentahydroxyflavon) and KNK437 (N-formyl-3,4-methylenedioxy-benzylidene-gamma-butyrolactam) are well known as inhibitors of thermotolerance and as heat-shock sensitizers (7,8). There are several studies showing antiproliferative and antiangiogenic properties of quercetin in cells (9-11). Previously, we also showed that quercetin and KNK437 have inhibitory effects on thermotolerance in prostate cancer cells. Additionally, we found that KNK437 and quercetin enhanced the cytotoxic and apoptotic effect of hyperthermia in these cells (12). To the best of our knowledge, there is no study examining the effects of KNK437 or quercetin on the angiogenesis of endothelial cells treated with hyperthermia. For this reason, we aimed to investigate the effects of hyperthermia, fractionated hyperthermia, and the mentioned agents on angiogenesis in human umbilical vein endothelial cells (HUVECs).

Materials and methods

Reagents

HUVECs and Matrigel™ were obtained from BD Biosciences (BD-354234, Two Oak Park, Bedford, MA, USA). EGM-2® BulletKit® (CC-3162, EBM-2 + supplements) for HUVEC culture was purchased from Lonza (Walkersville, MD, USA). KNK437 (*N*-formyl-3,4-methylenedioxy-benzylidene-γ-butyrolactam; Calbiochem, EMD Chemicals, Inc., USA) and quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one, Sigma Chemical Co., St Louis, MO, USA) were dissolved in dimethyl sulfoxide (DMSO) for use at the indicated concentrations.

Preparation of stock solutions of drugs

KNK437 (5 mg) was dissolved in 500 µL of DMSO (final concentration: ~ 4×10^4 µM). The stock solution was maintained at –20 °C for use at the indicated concentrations (50 and 100 µM). Stock solution was stable for 6 weeks at –20 °C.

Quercetin (14 mg) was dissolved in 1 mL of DMSO (final concentration: ~ 4×10^4 µM). The stock solution was maintained at +4 °C for use at the indicated concentrations (50 and 100 µM). Stock solution was stable for 12 weeks at +4 °C.

Cell culture

HUVECs were cultured in EGM-2 complete medium consisting of EBM-2 basal medium and supplements (ascorbic acid, hydrocortisone, heparin, GA-1000 and 2% FBS, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF-1)) except for serum starvation in which FBS concentration was reduced to 0.1% and no growth factors were added. Cell culture flasks or well plates were coated with gelatin (Sigma-Aldrich, Irvine, Ayrshire, UK). Cells were grown in a humidified atmosphere (95% air, 5% CO_2 at 37 °C) and passaged every 4-6 days. Cells between the fourth and the sixth passage were used for experiments. Cells were seeded in 6-well plates. When the cells reached 70%-80% confluency, they were treated with drugs and hyperthermia. The final concentration of DMSO in each culture medium was 0.25% (v/v), irrespective of the concentrations of the drugs. The same concentration of DMSO was used as a control.

Hyperthermia and drug treatments

All heating procedures were applied as described previously (7). Briefly, heat and drug treatments are indicated as follows:

37 °C and drug treatment:

1. Control group (C): Cells were incubated at 37 °C for 6 h with DMSO.
2. KNK437 group (KNK437): Cells were incubated at 37 °C for 6 h with 50 and 100 μM KNK437.
3. Quercetin group (Quercetin): Cells were incubated at 37 °C for 6 h with 50 and 100 μM quercetin.

Single hyperthermia and drug treatment:

1. Single hyperthermia group (SH): Cells were incubated at 37 °C for 5 h with DMSO and then heated at 45 °C for 1h.
2. KNK437 and single hyperthermia group (KNK437 + SH): Cells were incubated at 37 °C for 5 h with 50 and 100 μM KNK437 and then heated at 45 °C for 1 h.
3. Quercetin and single hyperthermia group (Quercetin + SH): Cells were incubated at 37 °C for 5 h with 50 and 100 μM quercetin and then heated at 45 °C for 1 h.

Fractionated hyperthermia and drug treatment:

1. Fractionated hyperthermia group (FH): Cells were incubated at 37 °C for 1 h with DMSO followed by heating at 45 °C for 10 min and then recovered at 37 °C for 4 h followed by heating at 45 °C for 1 h.
2. KNK437 and fractionated hyperthermia (KNK437 + FH): Cells were incubated at 37 °C for 1 h with 50 and 100 μM KNK437 followed by heating at 45 °C for 10 min and then recovered at 37 °C for 4 h followed by heating at 45 °C for 1 h.
3. Quercetin and fractionated hyperthermia (Quercetin + FH): Cells were incubated at 37 °C for 1 h with 50 and 100 μM quercetin followed by heating at 45 °C for 10 min and then recovered at 37 °C for 4 h followed by heating at 45 °C for 1 h.

In vitro angiogenesis assay

In vitro angiogenesis was assayed using Matrigel™ matrix (BD-354234, Two Oak Park, Bedford, MA, USA). Matrigel™ Basement Membrane Matrix is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma. EHS sarcoma is a tumor rich in extracellular matrix proteins including various growth factors, e.g., TGF-beta, epidermal growth factor, insulin-like growth factor, fibroblast growth factor, tissue plasminogen activator and other growth factors that occur naturally in the EHS tumor.

After being thawed at 4 °C, Matrigel™ matrix (250 μL) was quickly added to each well of a 24-well plate and allowed to solidify for 1 h at 37 °C. Hyperthermia and drug treated cells were added to each well at a density of ~2×10^4 cells/well in EGM-2 complete medium and incubated at 37 °C for 24 h. Tubular structures of HUVECs were visualized and photographed using an Olympus Photomicroscope (Olympus IX81S1F-2, Japan) with a ×4 lens. Tube lengths at 3 different areas were calculated as pixel values by means of UTHSCSA ImageTool software, version 3.0 (The University of Texas Health Science Center, San Antonio, Texas, USA). Experiments were carried out 3 times.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). We used Kolmogorov-Smirnov (one sample K-S) test from non-parametric tests to check whether data were in normal distribution or not. Comparison of groups according to the parameters was performed by one-way ANOVA in SPSS (IBM Corporation, Chicago, IL, USA). P values less than 0.05 were considered statistically significant.

Results

We found that KNK437 and quercetin (at 50 and 100 μM concentrations) suppressed the endothelial tube formations on Matrigel matrix at 37 °C as shown in Figure 1. The most effective agent was 100 μM KNK437 (Figure 1). Figure 2 shows that single hyperthermia at 45 °C for 1 h significantly ($P < 0.001$) decreased the tube length (10,366 ± 507) of endothelial cells on Matrigel. However, we found that tube lengths in the fractionated hyperthermia group (14,160 ± 466) were found to be significantly higher than those in the single hyperthermia group (Figure 2, $P < 0.01$). In both single hyperthermia and drug treatments group (Figure 3), both concentrations of KNK437 (50 μM ($P < 0.01$) and 100 μM ($P < 0.001$)) and 100 μM of quercetin ($P < 0.01$) decreased tube formations significantly. However, we did not find any significant difference between SH and quercetin (50 μM) + SH groups, or between KNK437 (50 μM) + SH and quercetin (50 μM) + SH. KNK437 (100 μM) was found to be the most effective agent for inhibition of angiogenesis in this experimental group (Figure 3).

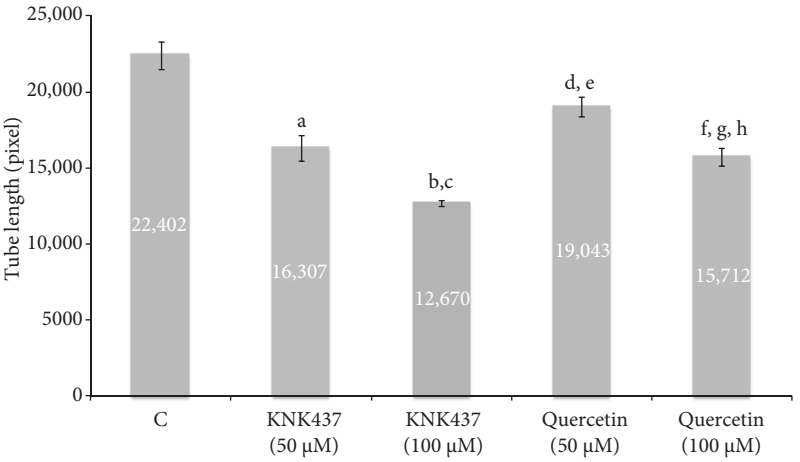

Figure 1. Tube formations of HUVECs on Matrigel in control (37 °C) and drug treatments. Bar graphs represent the tube lengths of control (C), KNK437, and quercetin (both at 50 and 100 μM concentrations). As mentioned in Materials and methods, tube lengths at 3 different areas were calculated as pixel values by means of UTHSCSA ImageTool Version 3.0 (The University of Texas Health Science Center, San Antonio, Texas, USA). Values represent means ± S.D. of triplicate samples per group. Significance values of $P < 0.05$ were represented as following; a: KNK437 (50 μM) vs. C; b: KNK437 (100 μM) vs. C; c: KNK437 (100 μM) vs. KNK437 (50 μM); d: Quercetin (50 μM) vs. C; e: Quercetin (50 μM) vs. KNK437 (50 μM); f: Quercetin (100 μM) vs. C; g: Quercetin (100 μM) vs. KNK437 (100 μM); h: Quercetin (100 μM) vs. Quercetin (50 μM).

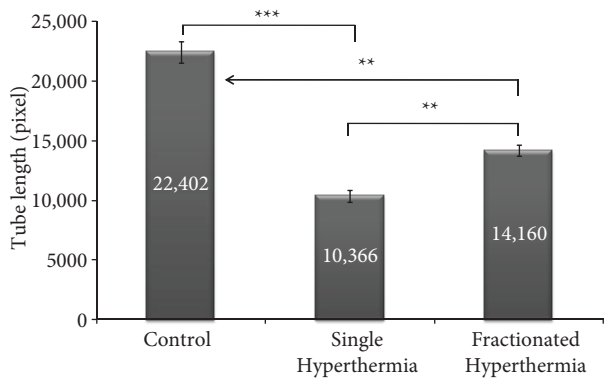

Figure 2. Tube lengths of HUVECs on Matrigel in control (C), single hyperthermia (SH) and fractionated hyperthermia (FH) groups. ***: $P < 0.001$, **: $P < 0.01$, *: $P < 0.05$. Pixel values represent means ± S.D. of tube lengths at 3 different areas.

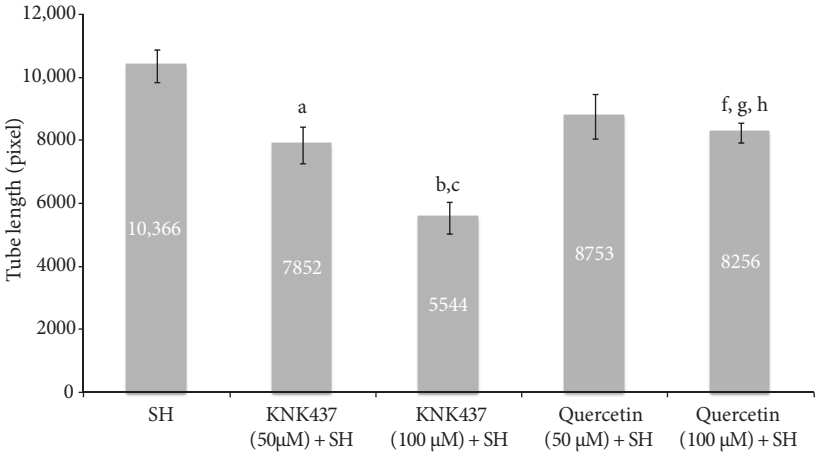

Figure 3. Tube formations of HUVECs on Matrigel in single hyperthermia and drug treatments. Bar graphs represent the tube lengths of single hyperthermia (SH), KNK437 (50 and 100 μM) + SH and quercetin (50 and 100 μM) + SH groups as pixel values at 3 different areas. Values represent means ± S.D. Significance values of $P < 0.05$ were represented as following; a: KNK437 (50 μM) + SH vs. SH; b: KNK437 (100 μM) + SH vs. SH; c: KNK437 (100 μM) + SH vs. KNK437 (50 μM) + SH; f: Quercetin (100 μM) + SH vs. SH; g: Quercetin (100 μM) + SH vs. KNK437 (100 μM) + SH; h: Quercetin (100 μM) + SH vs. Quercetin (50 μM) + SH.

In fractionated hyperthermia and drug treatments group (Figure 4), both KNK437 (50 μM, $P < 0.01$; 100 μM, $P < 0.001$) and quercetin (50 μM, $P < 0.01$; 100 μM, $P < 0.01$) decreased tube formations significantly. These results show that the increased capillary-like tube formation by fractionated hyperthermia was significantly suppressed by thermotolerance inhibitors in this group. There was no significant difference between KNK437 (50 μM) + FH and quercetin (50 μM) + FH groups in tube formations. We also did not find a difference between the quercetin (50 μM) + FH and quercetin (100 μM) + FH groups. The most effective blocking agent was found to be 100 μM KNK437 in this group (Figure 4).

Figure 5 shows the images of capillary-like tube formations of HUVECs in control (C), single hyperthermia (SH), fractionated hyperthermia (FH), KNK437, KNK437 + SH, and KNK437 + FH groups.

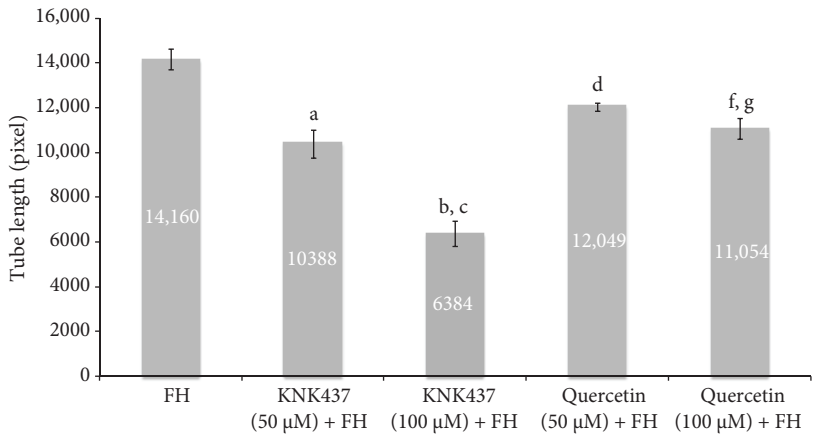

Figure 4. Tube formations of HUVECs on Matrigel in fractionated hyperthermia and drug treatments. Bar graphs represent the tube lengths of fractionated hyperthermia (FH), KNK437 (50 and 100 μM) + FH and Quercetin (50 and 100 μM) + FH groups as pixel values at 3 different areas. Values represent means ± S.D. Significance values of $P < 0.05$ were represented as following; a: KNK437 (50 μM) + FH vs. FH; b: KNK437 (100 μM) + FH vs. FH; c: KNK437 (100 μM) + FH vs. KNK437 (50 μM) + FH; d: Quercetin (50 μM) + FH vs. FH; f: Quercetin (100 μM) + FH vs. FH; g: Quercetin (100 μM) + FH vs. KNK437 (100 μM) + FH.

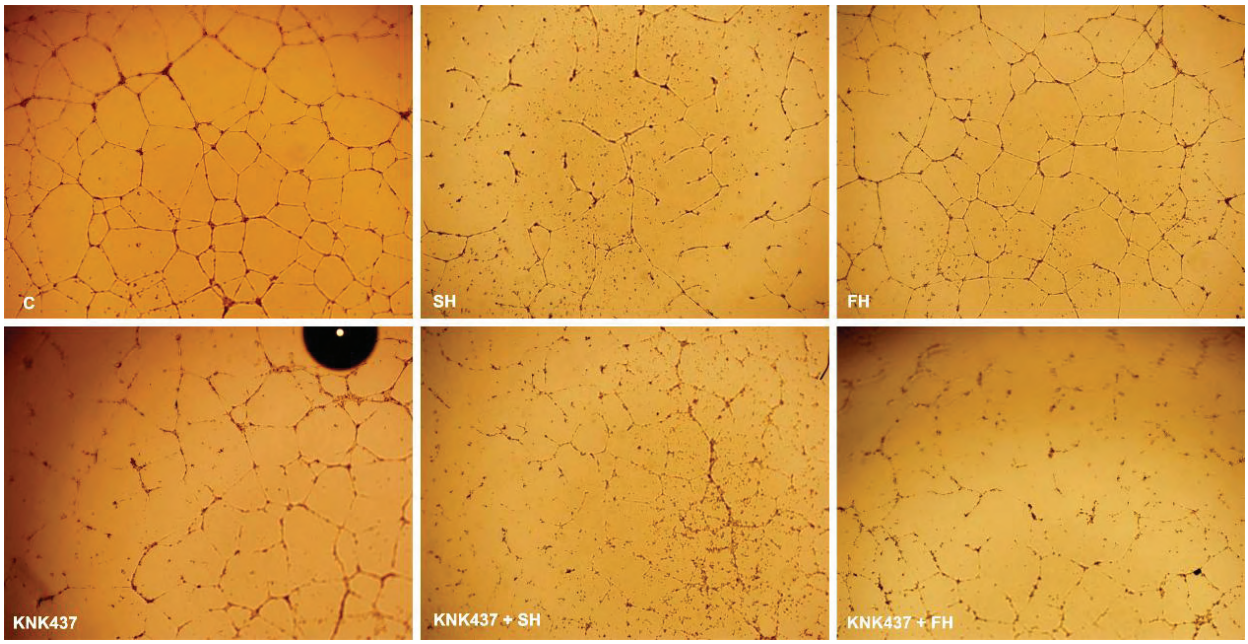

Figure 5. The representative images of capillary-like tube formations of HUVECs in control (C), single hyperthermia (SH), fractionated hyperthermia (FH), KNK437, KNK437 + SH and KNK437 + FH groups. Tubular structures of HUVECs were visualized and photographed using an Olympus Photomicroscope (Olympus IX81S1F-2, Japan) with a ×4 lens.

Discussion

Angiogenesis, which is the formation of new capillaries from preexisting vessels, plays an important role in tumor growth (13). Because the control of tumor angiogenesis is known to be independent from that of cancer cell proliferation (14), the use of agents that inhibit angiogenesis could be an important complementary treatment strategy to the traditional cancer therapies (15,16). In order to inhibit tumor angiogenesis, several strategies have been reported. These approaches include blockage of angiogenic proteins (VEGF; Bevacizumab®, bFGF) or their receptors (VEGFR or multiple growth factor receptors; Imatinib/Glivec®, Sunitinib/Sutent®, Sorafenib/Nexavar®), upregulation of endogenous inhibitors, or directly targeting tumor endothelial cells by apoptosis (17). In our previous study, we found that S-adenosyl methionine (DNA methylating agent) inhibited the proliferation, migration, and capillary-like tube formation of endothelial cells in vitro (18).

Hyperthermia is known to be an ideal therapy that has low toxicity, mild side effects, and has been shown to provide synergies with many of the traditional treatment modalities in cancer (3). The major problem in hyperthermia is the "thermotolerance" phenomenon. Hsps act as the main mediators of thermotolerance to protect cells against hyperthermia-induced damage. Hsp inhibitors, such as KNK437 and quercetin, are useful agents for the inhibition of thermotolerance (19). Additionally, in our previous study, both KNK437 and quercetin were shown to impede hyperthermia-induced thermotolerance and enhance hyperthermia-induced apoptosis in PC-3 cells (12). It has been shown that quercetin has antiproliferative activity on human fibroblasts, and endothelial and tumor cells (20,21). In addition, quercetin is reported to be a useful therapeutic agent for tumor angiogenesis (22).

In the present study, treatment of ECs with KNK437 and quercetin (at concentrations of 50 and 100 μM) at 37 °C significantly suppressed the endothelial tube formations on Matrigel matrix. Moreover, KNK437 (100 μM) was found to be the most effective agent in inhibiting angiogenesis. There is only one study supporting our finding about KNK437 (23). Our result suggests that KNK437 may be a valuable agent to attenuate tumor angiogenesis independent from hyperthermic therapy.

Several studies suggest that hyperthermia has antiproliferative and anti-angiogenic activities in endothelial cells and cancer cells (24,25). It is reported that hyperthermia inhibits angiogenesis and both endothelial cells and microvessels can be lethally damaged by the hyperthermia doses used as antineoplastic therapy (26). We found that single step hyperthermia (SH) (45 °C for 1 h) significantly decreased tube lengths in endothelial cells. Moreover, pretreatment of the SH group with KNK437 in concentration of 100 μM exhibited more potent activity against angiogenesis as a heat-sensitizer than quercetin because no differences were found between SH and quercetin (50 μM) + SH, or between KNK437 (50 μM) + SH and quercetin (50 μM) +SH groups in this study.

We also aimed to investigate whether the acquisition of thermotolerance is seen in angiogenesis using fractionated hyperthermia (FH) in endothelial cells. The tube lengths of the FH group (heat treatment at 45 °C for 10 min and second heating at 45 °C for 1 h after recovery at 37 °C for 4 h) appeared to be higher compared to the SH group, suggesting that thermotolerance developed in endothelial cells. In the FH group, pretreatment of cells with KNK437 or quercetin inhibited the acquisition of thermotolerance in angiogenesis. KNK437 was found to be more effective than quercetin in this inhibition because there were no significant differences between other groups as shown in Figure 4.

This study is important to stress the emphasis of hyperthermia in cancer therapy. Our study is original for suggesting the inhibitory effect of KNK437 on angiogenesis in both heat treated and non-heat treated endothelial cells. KNK437 pretreatment may be a useful approach in heat therapy because it is both angiogenesis and thermotolerance inhibiting. However, further studies are needed to explore the inhibitory mechanism of KNK437 with or without hyperthermia on angiogenesis.

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