Inhibition of heat shock protein 90 exerts an antitumour effect in angiosarcoma: involvement of the vascular endothelial growth factor signalling pathway

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Summary

Background Angiosarcoma is a rare malignant neoplasm derived from endothelial cells, and because advanced angiosarcoma is resistant to standard chemotherapy its prognosis is poor. Therefore, new therapies are urgently needed. Heat shock protein (HSP)90 has been identified as a molecular chaperone that regulates various cancer-related proteins. Numerous clinical trials are currently testing the effectiveness of HSP90 inhibitors in various types of malignancies.

Objectives To investigate the role of HSP90 in the pathogenesis of angiosarcoma and whether the inhibition of HSP90 may have antitumour activity.

Methods The expression of HSP90 protein in angiosarcoma was examined using immunohistochemistry and immunoblotting. The effects of HSP90 inhibition were proven using proliferation, migration and invasion assay in angiosarcoma cells. The mechanism of antitumour effect by HSP90 inhibition was investigated by the transfection of small interfering RNA (siRNA).

Results The levels of HSP90 protein expression in cultured angiosarcoma cell lines were markedly increased compared with those in normal tissue cell lines. Immunohistochemical analyses revealed that the expression of HSP90 protein was strongly detected in angiosarcoma tissues compared with that in normal dermal vessels or senile angioma tissues. Ganetespib, an HSP90 inhibitor, with or without taxanes, inhibited the proliferation of angiosarcoma cells via apoptosis in a dose-dependent manner. HSP90 siRNA suppressed the proliferation, migration and invasion in angiosarcoma cells. Knock-down of HSP90 did not suppress vascular endothelial growth factor receptor 2 directly, but selectively suppressed several downstream targets of vascular endothelial growth factor signalling in angiosarcoma cells.

Conclusions HSP90 could be a novel therapeutic target for angiosarcoma.

What’s already known about this topic?

- As advanced angiosarcoma is resistant to standard chemotherapy, its prognosis is poor and new therapies are urgently needed.
- Heat shock protein (HSP)90 has been identified as a molecular chaperone that regulates various cancer-related proteins. Numerous clinical trials are currently testing the effectiveness of HSP90 inhibitors in various types of malignancies.

What does this study add?

- HSP90 is overexpressed in angiosarcoma.
Angiosarcoma is a rare malignant tumour of endothelial origin. The 5-year survival rate of angiosarcoma is approximately 12–24% and its prognosis is poor compared with that of other malignant skin tumours. This is because angiosarcoma tends to spread widely and metastasize, which makes definitive therapies difficult.

Some agents reported to be efficacious for the treatment of angiosarcoma are paclitaxel, docetaxel, gemcitabine and bevacizumab. However, advanced angiosarcoma is frequently resistant to these agents, and new effective therapies need to be developed.

Heat shock protein (HSP)90 has attracted attention as a molecular target in cancer therapy. HSP90 is a molecular chaperone that functions in stabilizing and activating numerous client proteins required for the survival of cancer cells. HSP90α and HSP90β are two major cytoplasmic isoforms of HSP90. HSP90α is especially known as a stress-inducible protein. HSP90 and HSP90α are overexpressed in several malignant tumours and the inhibition of HSP90 suppresses tumour proliferation in vitro and in vivo. Numerous clinical trials are currently testing HSP90 inhibitors against different types of cancer. Ganetespib, a novel HSP90 inhibitor, exerts its competitive inhibitory effect by binding to the N-terminal ATP-binding site, and has been shown to have antitumour activity and lower hepatic and ocular toxicity compared with other HSP90 inhibitors. In addition, HSP90 inhibitors have attracted attention as sensitizers of cancer cells to both conventional chemotherapy and radiation therapy. Therefore, in our study, we investigated the role of HSP90 in the pathogenesis of angiosarcoma and whether the inhibition of HSP90 may have therapeutic potential.

Materials and methods

Reagents

Ganetespib was purchased from AdooQ BioScience (Irwin, CA, U.S.A.), and paclitaxel and docetaxel were purchased from Wako Pure Chemical Industries (Osaka, Japan). HSP90 small interfering RNA (siRNA) and control siRNA were purchased from Dharmacon (Lafayette, CO, U.S.A.). FITC Annexin V, propidium iodide (PI) solution, and Annexin V binding buffer were purchased from BioLegend (San Diego, CA, U.S.A.).

Tissue samples

In accordance with the Declaration of Helsinki, institutional review board approval and written informed consent were obtained before patients were entered into this study. Skin samples were obtained from the involved skin of four patients with angiosarcoma and four patients with senile angioma. Control skin samples were obtained from the skin of four healthy donors.

Cell cultures

We used four human cutaneous angiosarcoma cell lines, HAMON (passage 8–20), ISO-HAS (passage > 300), MO-LAS (passage > 300) and ISOS-1 (passage > 300). HAMON was kindly donated by Daichi Hoshina and Riichiro Abe. ISO-HAS, MO-LAS and ISOS-1 were kindly provided by Mikio Masuzawa. Primary human dermal microvascular endothelial cells (HDMECs) were obtained from Takara (Shiga, Japan). Human dermal fibroblasts (HDFs) were obtained by skin biopsy from healthy donors as described previously. The human immortalized keratinocyte cell line (HaCaT) was cultured as described previously.

Immunoblotting

Each of the antibodies are described in detail as follows: antibodies for HSP90 (1 : 500, Cell Signaling Technology, Beverly, MA, U.S.A.), vascular endothelial growth factor (VEGF) receptor (VEGFR)2 (1 : 1000, Cell Signaling Technology), phospho-VEGFR2 (1 : 1000, Cell Signaling Technology), mitogen-activated protein kinase (MEK) (1 : 1000, Cell Signaling Technology), phospho-MEK (1 : 1000, Cell Signaling Technology), HSP90α (1 : 1000, Abcam, Cambridge, U.K.), focal adhesion kinase (FAK) (1 : 1000, Abcam), phospho-FAK (1 : 1000, Abcam), paxillin (1 : 10000, Abcam), phospho-paxillin (1 : 20000, Abcam), vascular endothelial (VE) cadherin (1 : 1000, Abcam), phospho-VE cadherin (1 : 1000, Abcam), survivin (1 : 5000, Abcam), extracellular signal-regulated kinase (ERK) (1 : 1000, Cell Signaling Technology), phospho-ERK (1 : 1000, Cell Signaling Technology) and β-actin (1 : 500, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.).
U.S.A.). Immunoblotting was performed as described previously.\textsuperscript{27} The protein expression levels were quantitated by scanning densitometry.

**Immunohistochemical analysis**

Immunohistochemistry was performed with primary antibodies for HSP90 (1 : 50, Cell Signaling Technology) and HSP90\textsuperscript{α} (1 : 50, Abcam) as described previously.\textsuperscript{28}

**Growth inhibition assays**

The number of viable cells was determined using the cell counting kit (CCK)-8 assay (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer’s protocol. Each cell line was treated with six different concentrations (10 nmol L\textsuperscript{-1}, 25 nmol L\textsuperscript{-1}, 50 nmol L\textsuperscript{-1}, 75 nmol L\textsuperscript{-1}, 100 nmol L\textsuperscript{-1}, 1000 nmol L\textsuperscript{-1}) of ganetespib for 72 h. Control cells were treated with 0.1% dimethyl sulfoxide (DMSO). Combination effects of taxane and ganetespib were assessed with varying concentrations of taxane (paclitaxel or docetaxel at 50 nmol L\textsuperscript{-1}, 100 nmol L\textsuperscript{-1}, 250 nmol L\textsuperscript{-1}, 500 nmol L\textsuperscript{-1}, 750 nmol L\textsuperscript{-1} or 1000 nmol L\textsuperscript{-1}), either alone or in combination with the previously established half-maximal inhibitory concentration (IC\textsubscript{50}) concentrations of ganetespib. Control cells were also treated with 0.1% DMSO. Drug–drug interactions were evaluated using the combination index (CI) method.\textsuperscript{29} CI values represent the combination effect [synergistic (< 1), additive (= 1) or antagonistic (> 1)].

**Annexin V-FITC and propidium iodide assay for flow cytometry**

HAMON and ISO-HAS cells (1 × 10\textsuperscript{5}) were treated with ganetespib or 0.1% DMSO control. After 72 h treatment, Annexin V-FITC and PI assay was performed according to the manufacturer’s protocol. The samples were immediately measured using the FACSVerse flow cytometer (BD Biosciences, San Jose, CA, U.S.A.) and analysed by the FACSuite\textsuperscript{®} software (BD Biosciences).

**Transient transfection**

Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, U.S.A.) was used as the transfection reagent according to the manufacturer’s protocol.

**Cell viability count**

After transfection with control or HSP90 siRNA for 72 h, viable HDMEC, HAMON and ISO-HAS cells were stained with trypan blue (Sigma Aldrich, St. Louis, MO, U.S.A.) and counted using the TC20\textsuperscript{®} Automated Cell Counter (Biorad, Berkeley, CA, U.S.A.).

**Cell migration assay and cell invasion assay**

Migration and invasion assays were performed as described previously.\textsuperscript{10} The number of transmembrane cells was counted under a microscope at original magnification × 100.

**Caspase 3/7 assays**

HAMON and ISO-HAS cells were transfected with control or HSP90 siRNA. At 72 h post-transfection, caspase-3/7 activity was determined using a Caspase-Glo 3/7 kit (Promega Corporation, Madison, WI, U.S.A.) according to the manufacturer’s protocol.

**Statistical analysis**

Data were presented as bar graphs that showed the mean ± SD of at least three independent experiments. The statistical analyses were performed using the Mann–Whitney U-test for comparison of medians. A \(P\)-value < 0.05 was considered to be statistically significant.

**Results**

**Heat shock protein 90 is overexpressed in angiosarcoma**

We performed immunohistochemical staining to compare the expression of HSP90 and HSP90\textsuperscript{α} in angiosarcoma tissues with senile angioma (benign vascular tumour) tissues and normal vessel tissues. The typical immunostaining intensity of HSP90 and HSP90\textsuperscript{α} is shown in Figure 1b. The HSP90- and HSP90\textsuperscript{α}-stained tissues were categorized according to their immunoreactivity [(--) – (++++)]. HSP90 and HSP90\textsuperscript{α} were statistically more strongly expressed in angiosarcoma tissues than in senile angioma tissues and normal vessels (Fig. 1a, c, d). Next, we used Western blotting to examine the expression of HSP90 and HSP90\textsuperscript{α} in angiosarcoma cell lines (HAMON, ISO-HAS, MO-LAS and ISOS-1). Western blotting showed that the levels of HSP90 and HSP90\textsuperscript{α} in angiosarcoma cell lines were markedly increased compared with normal tissue cell lines (HDMEC, HaCaT and HDF) (Fig. 1e–g).

**Inhibition of heat shock protein 90 reduces the proliferation of angiosarcoma cells**

We investigated whether inhibition of HSP90 was effective in HAMON and ISO-HAS. After a 72-h treatment with six different concentrations (10 nmol L\textsuperscript{-1}, 25 nmol L\textsuperscript{-1}, 50 nmol L\textsuperscript{-1}, 75 nmol L\textsuperscript{-1}, 100 nmol L\textsuperscript{-1}, 1000 nmol L\textsuperscript{-1}) of the HSP90 inhibitor ganetespib, cell viability was measured using the CCK-8 assay. Ganetespib significantly inhibited the proliferation of angiosarcoma cells in addition to HDMEC, in a dose-dependent manner (Fig. 2a). The IC\textsubscript{50} values for ganetespib at 72 h were 43.5 nmol L\textsuperscript{-1} in HDMEC, 84.8 nmol L\textsuperscript{-1} in HAMON and 82.7 nmol L\textsuperscript{-1} in ISO-HAS cells (Table 1).
Next, we used Annexin V and PI staining for flow cytometry to assess whether ganetespib induced apoptosis in angiosarcoma cells. As shown in Figure 2b, ganetespib (IC\text{50} concentrations at 72 h) enhanced apoptosis in HAMON and ISO-HAS cells compared with negative controls, as demonstrated by the increased number of ganetespib-treated Annexin V-positive cells. In HAMON cells, the early apoptotic percentage (lower right) of the control group was 33.8%, while in the ganetespib group this figure was 59.67%. Similarly, in ISO-HAS cells, the early apoptotic percentage of the control group was 24.76%, while in the ganetespib group this figure was 52.38%. These results indicate that ganetespib downregulated the proliferation of angiosarcoma cells via apoptosis.

**Combination therapy with ganetespib and taxane is effective in angiosarcoma cells**

In clinical practice, taxanes are an effective therapy for patients with angiosarcoma.\textsuperscript{3,4} Paclitaxel has been reported
to inhibit the proliferation of angiosarcoma cell lines, such as ASM and ISO-HAS, in an experimental setting. Thus, we analysed the inhibitory effect of taxane monotherapy, and also combination therapy with ganetespib and taxane, in angiosarcoma cells.

Both paclitaxel and docetaxel showed a significant inhibitory effect in angiosarcoma cells in a dose-dependent manner (Fig. 2c). The IC₅₀ values for paclitaxel at 72 h were 140.8 nmol L⁻¹ in HAMON and over 1000 nmol L⁻¹ in ISO-HAS cells. The IC₅₀ values for docetaxel at 72 h were 345 nmol L⁻¹ in HAMON and over 1000 nmol L⁻¹ in ISO-HAS cells (Table 1).

IC₅₀ values for taxanes were significantly decreased in combined therapy with ganetespib compared with taxane monotherapy (Fig. 2c, Table 1). In HAMON cells, the IC₅₀ values ranged from 140.8 nmol L⁻¹ (paclitaxel monotherapy) to 35.2 nmol L⁻¹ (paclitaxel plus ganetespib), and from 345 nmol L⁻¹ (docetaxel monotherapy) to 290.4 nmol L⁻¹ (docetaxel plus ganetespib). Similarly, in ISO-HAS cells, the IC₅₀ values ranged from over 1000 nmol L⁻¹ (paclitaxel monotherapy) to 878.8 nmol L⁻¹ (docetaxel plus ganetespib).

To evaluate whether the interaction of combined drugs was synergistic, additive or antagonistic, cell lines were exposed to both drugs at nonconstant concentration ratios for 72 h. As shown in Figure 2d, most of the combination groups were below the additivity line, indicating that these treatments had a synergistic effect. On the other hand, the CI value of combination treatment with ganetespib and docetaxel in HAMON was 1.00 ± 0.09, which indicated that this treatment had an additive effect. Based on these results, the combination treatment of a taxane with ganetespib is synergistic or additive in angiosarcoma cells.

**Heat shock protein 90 small interfering RNA inhibits proliferation, migration and invasion in angiosarcoma cells**

To clarify the role of HSP90 in the pathogenesis of angiosarcoma, we examined the effect of HSP90 siRNA in...
angiosarcoma cell lines compared with HDMEC. The level of downregulation of HSP90 expression by siRNA is shown in Figure 3a. Transfection of HSP90 siRNA significantly decreased the proliferation of angiosarcoma cells. In contrast, transfection of HSP90 siRNA did not affect that of HDMEC (Fig. 3b–d). These results were consistent with the expected effect of HSP90 inhibitor in angiosarcoma.

Next, we assessed the effect of HSP90 siRNA on the migration and invasion of angiosarcoma cells. Cell migration/invasion assay demonstrated that HSP90 siRNA significantly inhibited migration and invasion in angiosarcoma cells (Fig. 3e–h). These results indicate that knock-down of HSP90 induces an inhibitory effect on angiosarcoma cell growth, migration and invasion.

Knock-down of heat shock protein 90 did not suppress vascular endothelial growth factor receptor 2 directly, but selectively suppressed the downstream targets of vascular endothelial growth factor signalling in angiosarcoma cells

VEGF signalling is an important pathway involved in the angiogenesis and proliferation of angiosarcoma cells. Thus, to clarify the mechanism by which the knock-down of HSP90 exerts its antitumour activity, we examined whether HSP90 knock-down affected VEGFR2 phosphorylation in angiosarcoma. As shown in Figure 4a, HSP90 knock-down did not directly inhibit VEGFR2 phosphorylation.

Accordingly, we further examined whether HSP90 knock-down affected the downstream of VEGF signalling molecules.
such as ERK, FAK and survivin. HSP90 siRNA decreased ERK phosphorylation (Fig. 4b), while the phosphorylation of MEK showed little change (Fig. 4c). These results suggested that HSP90 inhibition directly contributes to ERK phosphorylation without affecting MEK phosphorylation in angiosarcoma. Knock-down of HSP90 significantly suppressed the phosphorylation of FAK and paxillin in Figure 4d. Moreover, HSP90 siRNA inhibited VE cadherin phosphorylation (Fig. 4e). Furthermore, HSP90 siRNA suppressed the expression of survivin (Fig. 4f) and enhanced the activity of caspase 3/7 in angiosarcoma cells (Fig. 4g). Taken together, our results demonstrate that the inhibition of HSP90 exerts its antitumour effect not

**Table 1** Summary of half-maximal inhibitory concentration (IC\textsubscript{50}) values for angiosarcoma cell lines HAMON and ISO-HAS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HAMON (nmol L\textsuperscript{-1})</th>
<th>ISO-HAS (nmol L\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ganetespib</td>
<td>84.8</td>
<td>82.7</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>140.8</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Ganetespib (IC\textsubscript{50} + paclitaxel)</td>
<td>35.2</td>
<td>23.7</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>345</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Ganetespib (IC\textsubscript{50} + docetaxel)</td>
<td>290.4</td>
<td>878.8</td>
</tr>
</tbody>
</table>
Fig 3. Knock-down of heat shock protein 90 (HSP90) inhibits cell growth, migration and invasion in angiosarcoma. (a) The levels of downregulation of HSP90 expression by small interfering RNA (siRNA). Human dermal microvascular endothelial cells (HDMECs) (b), HAMON (c, e, g) and ISO-HAS cells (d, f, h) were transfected with control or HSP90 siRNA. After treatment for 72 h, we evaluated the cell proliferation, migration and invasion activity. Data represent the mean and SD from three independent experiments. (b–d) The number of angiosarcoma cells was counted using a particle counter. (e, f) Cell migration was evaluated using transwell inserts coated without matrigel. (g, h) Cell invasion was evaluated using transwell inserts coated with matrigel. Haematoxylin and eosin staining for angiosarcoma cells treated with control or HSP90 siRNA. Original magnification × 100. *p < 0.05 compared with the values with control siRNA (1-0).
by targeting VEGFR2 directly, but by selectively targeting several downstream targets of VEGF signalling.

**Discussion**

Anthracyclines, ifosfamide and taxanes, either singly or in combination regimens, are commonly used to treat angiosarcoma. Comorbidities and the risk of adverse events often reduce the effectiveness of chemotherapy because most patients with angiosarcoma are elderly. As an effective therapy for treating angiosarcoma has not yet been established, there is a need to develop new therapeutic agents.

In this study, we demonstrated that HSP90 was overexpressed in angiosarcoma and the inhibition of HSP90 was effective in inhibiting the proliferation, migration and invasion of angiosarcoma cells. Moreover, we have shown that the knock-down of HSP90 exerted its anticancer effect by inhibiting the downstream targets of the VEGF signalling pathway.

HSP90 is a molecular chaperone protein that is essential for the maintenance and progression of cancer. In addition, overexpression of HSP90 is reported in several malignancies and is correlated with a poor prognosis. Our results showed that HSP90 was overexpressed in angiosarcoma cell lines in vitro and in tissues derived from patients with angiosarcoma.

Ganetespib, an HSP90 inhibitor, has been shown to induce growth arrest and apoptosis in various cancer models. In several clinical trials, ganetespib has not only demonstrated an antitumour effect, but also caused few side-effects.

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**Fig 4.** Knock-down of heat shock protein (HSP)90 inhibits the downstream of vascular endothelial growth factor (VEGF) signalling. HAMON and ISO-HAS were transfected with control or HSP90 siRNA. (a–f) After 72 h, the expression of phosphorylated VEGF receptor (VEGFR)2, total VEGFR2 (a), phosphorylated extracellular signal-regulated kinase (ERK), total ERK (b), phosphorylated mitogen-activated protein kinase (MEK), total MEK (c), phosphorylated focal adhesion kinase (FAK), total FAK, phosphorylated paxillin, total paxillin (d), phosphorylated vascular endothelial (VE) cadherin, total VE cadherin (e), survivin (f) and β-actin levels were analysed by immunoblotting. The results of one experiment representative of three independent experiments are shown. The ratio of phosphorylated VEGFR2 to total VEGFR2 (a), phosphorylated ERK to total ERK (b), phosphorylated MEK to total MEK (c), phosphorylated FAK to total FAK, phosphorylated paxillin to total paxillin (d), phosphorylated VE cadherin to total VE cadherin (e) and survivin to β-actin (f) were quantified by scanning densitometry. Relative levels are shown in HAMON and ISO-HAS treated with HSP90 siRNA compared with control. *P < 0.05 compared with the values in control. (g) After 72 h post-transfection, caspase-3/7 activity was examined using Caspase-Glo 3/7 kit. n.s., not significant.
Fig 4. Continued.

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Furthermore, ganetespib combined with docetaxel has significantly extended progression-free and overall survival in several patients with advanced nonsmall cell lung cancer. It is consistent with our results that ganetespib combined with taxanes had a synergistic antiproliferative effect in angiosarcoma cells. On the other hand, ganetespib inhibited the proliferation of HDMEC, whereas HSP90 siRNA used to knock-down HSP90 expression selectively did not affect HDMEC. These results may imply that increasing the selectivity of HSP90 inhibitor is essential for minimizing adverse effects, although there has been no report on severe cardiovascular events in relation to the use of ganetespib in clinical trials.

VEGF has been shown to play an essential role in promoting angiogenesis. Additionally, angiosarcoma has been shown to be dependent on VEGF signalling. However, anti-VEGF monoclonal antibodies and other VEGFR inhibitors, such as bevacizumab, sunitinib and sorafenib had little efficacy in angiosarcoma. In addition, bevacizumab was not effective in cultured angiosarcoma cell lines in vitro and tumour growth in vivo. These reports imply that downstream targets of VEGF signalling may be more important than VEGF/VEGFR as therapeutic targets for the treatment of angiosarcoma. Our results reveal that the knock-down of HSP90 did not directly suppress VEGFR2, but selectively suppressed the downstream targets of VEGF signalling in angiosarcoma cells. The MEK/ERK pathway is critical for cell proliferation. Additionally, FAK and paxillin regulate the migration and invasion of cancer cells. FAK facilitates the phosphorylation of VE cadherin and endothelial cell junctional breakdown. Moreover, survivin inhibits apoptosis, and caspase 3/7 are essential for cell death. We demonstrated that HSP90 siRNA affected the downstream of VEGF signalling molecules, which leads to suppressed cell growth and induces metastasis suppression and apoptosis in angiosarcoma. Thus, our results suggest that HSP90 inhibition exerts its anticancer effect on
angiosarcoma cells by blocking the various downstream targets of VEGF signalling (Fig. 5).

Although HSP90 is reported to be essential for VEGFR2 activation, which is one of the HSP90 client proteins, our results showed that HSP90 knock-down did not affect VEGFR2 phosphorylation in angiosarcoma cell lines. In previous reports, the effects of HSP90 inhibitor on client proteins differed depending on the cell line in liposarcoma and pancreatic cancer. Based on these findings, our results indicate that the contribution of HSP90 to client proteins varies according to the type of cell, i.e. endothelial cells or angiosarcoma cells.

In this study, we demonstrated that HSP90 inhibition had an antitumour effect in angiosarcoma cells. The effectiveness of HSP90 inhibitors for angiosarcoma needs to be assessed in vivo and in clinical trials, which was a limitation of our study. Further studies could bring HSP90 inhibition closer to the goal of clinical use. We conclude that the inhibition of

Fig 4. Continued.

Fig 5. A hypothetical model of the antitumour effect of heat shock protein (HSP)90 inhibitor in angiosarcoma. ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase.
HSP90 could be a potential therapeutic target for the treatment of angiosarcoma.

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