A pyrrole-based natural small molecule mitigates HSP90 expression in MDA-MB-231 cells and inhibits tumor angiogenesis in mice by inactivating HSF-1

K. C. Rashmi¹ · H. S. Atreya² · M. Harsha Raj¹ · Bharathi P. Salimath¹ · H. S. Aparna¹

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Abstract Heat shock proteins (HSPs), molecular chaperones, are crucial for the cancer cells to facilitate proper functioning of various oncoproteins involved in cell survival, proliferation, migration, and tumor angiogenesis. Tumor cells are said to be “addicted” to HSPs. HSPs are overexpressed in many cancers due to upregulation of transcription factor Heat-shock factor 1 (HSF-1), the multifaceted master regulator of heat shock response. Therefore, pharmacological targeting of HSPs via HSF-1 is an effective strategy to treat malignant cancers like triple negative breast cancer. In the current study, we evaluated the efficacy of a pyrrole derivative [bis(2-ethylhexyl)1H-pyrrole-3,4-dicarboxylate], TCCP, purified from leaves of *Tinospora cordifolia* for its ability to suppress heat shock response and angiogenesis using MDA-MB-231 cells and the murine mammary carcinoma: Ehrlich ascites tumor model. HSP90 was downregulated by TCCP by inactivation of HSF-1 resulting in inhibition of tumor cell proliferation, VEGF-induced cell migration, and concomitant decrease in tumor burden and neo-angiogenesis in vivo. The mechanism of suppression of HSPs involves inactivation of PI3K/Akt and phosphorylation on serine 307 of HSF-1 by the activation of ERK1. HSF-1 and HSP90 and 70 localization and expression were ascertained by immunolocalization, immunoblotting, and qPCR experiments. The anti-angiogenic

effect of TCCP was studied in vivo in tumor-bearing mice and *ex vivo* using rat corneal micro-pocket assay. All the results thus corroborate the logic behind inactivating HSF-1 using TCCP as an alternative approach for cancer therapy.

Keywords Pyrrole · *Tinospora cordifolia* · NMR · HSF-1 · HSP · MDA-MB-231

Abbreviations

HSF-1	Heat-shock factor 1
HSP	Heat shock protein
TCCP	<i>Tinospora cordifolia</i> compound pyrrole
LC-MS	Liquid chromatography–mass spectrometry
NMR	Nuclear magnetic resonance
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
HRP	Horseradish peroxidase
PVDF	Polyvinylidene fluoride
ECL	Enhanced chemiluminescence
DMSO	Dimethyl sulfoxide
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

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✉ H. S. Aparna
has.uom@gmail.com

¹ Department of Studies in Biotechnology, University of Mysore, Mysuru, Karnataka 570 006, India

² NMR Research Centre, Indian Institute of Science, Bengaluru 560 012, India

Introduction

In spite of early detection and novel therapeutic interventions, metastatic breast cancer is still the leading cause of cancer death in women. Triple negative breast cancer (TNBC) accounts for about 10–15% of all breast cancers, characterized by absence of all three molecular markers, namely, estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (Brewster et al. 2014). TNBC is aggressive and is associated with high mortality rate compared to other

subtypes since it has limited therapeutic options and hence there is a need for newer targeting strategies and drugs.

Heat shock proteins (HSPs) are molecular chaperones that are evolutionarily conserved and participate in stabilizing and activating more than 200 client proteins of which many are imperative for stress adaptation and constitutive cell signaling (Wandinger et al. 2008). Cancer cells depend heavily on the HSP90 chaperone machinery that comprises HSP90, HSP70, and other co-chaperones to prevent the misfolding and degradation of all the mutated and overexpressed oncoproteins (Trepel et al. 2010). Accordingly, HSPs are overexpressed in many cancers; specifically, HSP90 and HSP70 are reportedly overexpressed in breast cancer cells (Chiosis et al. 2013; Du et al. 2014). HSP90 promotes the oncogene addiction for the survival of cancer cells (Trepel et al. 2010). Overexpression of HSP90 and HSP70 in breast cancer as a result of heat shock response (HSR) owing to the proteotoxic stressors constitutively activates heat shock factor-1 (HSF-1), a transcription factor that drives neoplastic transformation by modulating diverse pathways that regulate cell survival, proliferation, protein synthesis, and cellular metabolism (De Thonel et al. 2011). HSF-1 overexpression often correlates to poor prognosis of breast cancer patients. HSF-1 translocates into the nucleus upon trimerization in the cytosol and binds to the heat shock response element (HSRE) in the promoter region on *hsp* genes. However, the activation of HSF-1 is regulated by posttranslational modifications such as phosphorylation, sumoylation, and deacetylation (Ciocca et al. 2013). Normally, HSF-1 shuttles between cytoplasm and nucleus, but upon activation by stress in a tumor cell, it concentrates in the nucleus (Dai et al. 2012). Phosphorylation on Ser²³⁰, Ser³²⁰, Ser³²⁶, and Thr¹⁴² residues by CaMKII, PKA, mTORC1, and CK2 respectively activates HSF-1 (Santagata et al. 2011b). On the contrary, HSF-1 is repressed as a result of phosphorylation on Ser¹²¹, Ser³⁰³, Ser³⁰⁷, and Ser³⁶³ residues by MK2 kinase, GSK3b, ERK, and JNK/SAPK or PKC, respectively. In the breast cancer cell lines, HSF-1 is directly phosphorylated by Akt and regulates its stability, transcriptional activity, and intracellular trafficking. Akt is usually dysregulated and hyperactive in human tumors and thus becomes an important target for cancer therapeutics. Besides regulating the expression of HSPs, HSF-1 has also been shown to regulate tumor angiogenesis and metastasis. Knockdown of *HSF-1* strongly suppresses accumulation of hypoxia-inducible factor 1- α (HIF-1 α) and also vascular endothelial growth factor (VEGF) both in vitro and in vivo (Gabai et al. 2012).

Considering hypoxic conditions experienced by growing tumor cells, there is increased production of VEGF-A₁₆₅, the most abundant and physiologically potent cytokine that stimulates tumor angiogenesis by binding to its receptor 2 (VEGFR2, KDR, Flk-1) to provide oxygen and nutrients to the growing tumor (Carmeliet and Jain 2011).

HSP90 inhibitor geldanamycin degrades the mature plasma membrane VEGFR2 in primary endothelial cells resulting in

an acute inhibition of VEGF signaling. HSP90 inhibition resulted in increased VEGFR2-HSP70 complex formation, leading to caltherin-mediated endocytosis and degradation of the receptor in the endosome-lysosome system. This HSP-mediated inhibition of angiogenesis was assessed in vivo by the decreased endothelial cell regeneration in the femoral artery repair of a mouse model and also impaired angiogenesis in zebra fish model (Bruns et al. 2012). Furthermore, overexpression of HSF-1 significantly correlated with tumor angiogenesis in patients with NSCLC (Cui et al. 2015). Likewise, *HSF-1* has also been identified as one among six potent metastasis-promoting genes (Scott et al. 2011) and is associated with potentiating metastasis in hepatocellular (Fang et al. 2012), prostate (Hoang et al. 2000; Tang et al. 2005), breast, colon, and lung cancers (Mendillo et al. 2012). For the past two decades, targeting HSP90 inhibition, in order to disturb the protein homeostasis, was presumed to be a good therapeutic option for cancer treatment. Various HSP90 inhibitors such as 17-AAG (tanespimycin), 17-DMAG (alvespimycin), and 13 more have been studied in clinical trials on cancer patients (Trepel et al. 2010). However, inhibition of HSP90 by inhibitors like geldanamycin does not suffice in cancer treatment since inhibition of HSP90 upregulates expression of HSP70 that maintains the chaperone activity. Thus, targeting overall HSP expression by directly inactivating HSF-1 is a promising strategy in anti-cancer drug discovery and therapy.

Compounds like triptolide, quercetin, benzylidene lactam, and emunin have been reported as HSF-1 inhibitors (Whitesell and Lindquist 2009). Also, thiazole nucleoside analogues, ritonavir, sulforaphane, and phenethyl isothiocyanate reduced HSF-1 expression in pancreatic cancer cells (Xia et al. 2012), renal cancer cells (Sato et al. 2012), and breast cancer cells (Sarkar et al. 2012). However, KRIBB11, a cell-permeable 2,6-diaminopyridine compound, is a more authentic HSF-1 inhibitor. Its interaction with HSF-1 is reversible and blocks the transcription of HSPs and is reported to have anti-proliferative activity in cancer cell lines and regressed HCT116 tumor growth in mice (Yoon et al. 2011). High-throughput screening for new HSF-1 inhibitors have yielded promising lead molecules (limonoids, curvularins, withanolides, celastrols, and colletotragarones) with potent HSF-1 inhibitory capability, but they lack specificity (Santagata et al. 2011b, 2013).

In the present investigation, we report the isolation, purification, and characterization of a novel bioactive small molecule, TCCP, a pyrrole derivative [bis(2-ethylhexyl)1H-pyrrole-3,4-dicarboxylate], from leaves of *Tinospora cordifolia*. Extensive research has been carried out on *T. cordifolia* (Wild.) Miers, Family: Menispermaceae, also called heartleaf moonseed or heavenly elixir (English), a large glabrous climber grown in tropical and subtropical regions of India for its potential medicinal properties such as anti-diabetic, anti-periodic, anti-spasmodic, anti-inflammatory, anti-arthritic, anti-allergic, anti-stress, anti-leprotic, anti-malarial, antioxidant,

antibacterial, and hepatoprotective properties (Ittiyavirah and Rahees 2013; Mittal et al. 2014). Various reports have also reported anti-neoplastic, anti-tumor, anti-angiogenic (Leyon and Kuttan 2004; Thippeswamy et al. 2008; Leyon and Kuttan 2004; Thippeswamy et al. 2008), and anti-metastatic activities in various in vivo models (Adhvaryu et al. 2008; Jagetia and Rao 2006; Leyon and Kuttan 2004; Singh et al. 2005; Thippeswamy and Salimath 2007; Thippeswamy et al. 2008). Overall, pharmacological inhibition of HSF-1 in turn inhibits HSPs and various proteins down the cancer induction cascade like pro-angiogenic regulators that may in turn down-regulate a multitude of signaling pathways characteristic of neoplastic cells (Chiosis et al. 2013; Du et al. 2014).

Our experiments validated TCCP's ability to downregulate HSPs by inactivating HSF-1 in the triple negative breast cancer cell line MDA-MB-231 in vitro, and the anti-HSP, anti-angiogenic, and anti-proliferative effects were also studied in vivo using the Ehrlich ascites murine mammary carcinoma model (EAT model). Furthermore, the mechanisms and signaling pathways involved in TCCP-mediated HSP downregulation was investigated. Our data suggest that TCCP is a potential drug candidate for the treatment of triple negative breast cancer.

Material and methods

Cell lines, tumor model, and chemicals

MDA-MB-231 cells were procured from the National Centre for Cell Science, Pune, India. Dulbecco's modified Eagle's medium, fetal bovine serum (FBS) of South American origin, and penicillin-streptomycin mixture and phosphate-buffered saline (PBS) without calcium and magnesium were purchased from Gibco, Life Technologies, USA. Recombinant human vascular endothelial growth factor (VEGF-A₁₆₅) was expressed and purified from pET3d VEGF₁₆₅ plasmid. 17-(Allylamino)-17-demethoxygeldanamycin (17-AAG) used as positive control was procured from Sigma-Aldrich. Wistar rats (4–6 months old) and female Swiss albino mice (8–10 weeks) were obtained from the animal house, University of Mysore, Mysore, India, with the permission of animal ethics committee (No. 122/GO/ReBi/S/99/CPCSEA). Ehrlich ascites tumor (EAT) cells were maintained in our laboratory by intraperitoneal transplantation as previously described (Gururaj et al. 2003). HSF-1 (#4356), pAKT-Ser⁴⁷³ (#9271), AKT (#9272), GAPDH (#2118), and Lamin B1 (#13435) antibodies were procured from Cell Signaling Technologies, and pHSF-Ser³⁰⁷ (sc-135640), pERK^{1/2} (sc-16982), and ERK^{1/2} (sc-292838) antibodies were from Santa Cruz Biotechnology. HSP90 (SAB1305541) and HSP70 (H5147) monoclonal antibodies were from Sigma-Aldrich, USA. Anti-rabbit IgG-HRP (621140380011730) and Atto

488 (18772) tagged secondary antibodies were from Merck Biosciences and Sigma-Aldrich, respectively. All other chemicals and reagents were of highest analytical grade.

Extraction and isolation

Leaves of *T. cordifolia*, collected from Western Ghats, India, was identified and authenticated by taxonomist Dr. Sudarshan, and a voucher specimen was deposited at the Department of Botany, University of Mysore, Mysuru. Shade-dried and powdered leaves were extracted by polarity-based Soxhlet extraction. Based on the bioactivity profile, the butanolic fraction among all the others was freed from solvent and used for further analysis.

Column chromatography

Fractionation of butanolic extract was performed on an activated silica gel column (35 × 2 cm) using ethyl acetate and methanol (2:1, v/v) as the eluent. The flow rate was maintained at 0.2 mL/min, and the fractions collected were tested by HPTLC (Merck) using toluene, ethyl acetate, chloroform, and acetic acid (6:2:5:2, v/v). Fractions with identical spots and R_f values were pooled and concentrated for further analysis.

Characterization

The purified fraction was subjected to LC-MS analysis (Waters, USA) and IR spectroscopy (Thermo-Nicolet 6700 spectrometer). One- and two-dimensional NMR spectra were acquired on an Agilent NMR spectrometer (400MR DD2) operating at ¹H resonance frequency of 400 MHz. NMR spectra were recorded at 25 °C in DMSO-d₆ as a solvent. Chemical shifts were quoted with respect to tetramethylsilane (TMS) as an external standard.

Trypan blue dye exclusion assay

An in vitro study on TCCP treatment-induced cytotoxicity was performed on MDA-MB-231 cells to determine the IC₅₀ value. Briefly, cells (3 × 10⁴) were seeded in 12-well plates (Nunc, USA) and incubated overnight at 37 °C and 5% CO₂. Post treatment with vehicle (0.1% DMSO), 17-AAG (1 to 20 μM/mL), and TCCP (10 to 100 μM/mL) for 12, 24, and 48 h, cells were rinsed in PBS, trypsinized, stained with trypan blue, and counted using a hemocytometer. Vehicle-treated viable (100%) cells were considered to calculate the half maximal (50%) inhibitory concentration (IC₅₀) of TCCP and 17-AAG-treated cells using GraphPad Prism 5.0.

MTT assay

MDA-MB-231 cells growing in the exponential phase were seeded into 96-well plates (Nunc MicroWell™) in triplicates at 3×10^4 cells per well in 100 μ L in complete medium. Increasing concentrations of TCCP were added (10 to 100 μ M/mL) and incubated for 24 h along with vehicle-treated (0.1% DMSO) control and 17-AAG-treated (5 μ M) positive control. Later, 10 μ L MTT (5 mg/mL) was added and incubated for 4 h at 37 °C. The resultant formazan was dissolved in 100 μ L DMSO and absorbance measured at test and reference wavelengths of 570 and 630 nm (Varioskan™ Flash Multimode Reader, Thermo Scientific).

Immunolocalization

Immunofluorescence staining to study the intracellular trafficking and localization of HSF-1, HSP90, and HSP70 was performed as described earlier (Lucas et al. 2010). Briefly, MDA-MB-231 cells (3×10^4) grown on coverslips were treated with TCCP (25 and 50 μ M), 17-AAG (5 μ M), or vehicle (0.1% DMSO) and incubated for 24 h. Followed by PBS wash, cells were fixed in 3% paraformaldehyde, permeabilized using 0.01% Triton X-100/PBS, blocked in 3% BSA for 30 min, and washed with PBS. The cells were then incubated with either anti-HSP90, HSP70, or HSF-1 antibody (1:500) for 1 h, washed, and probed with anti-rabbit IgG-Atto 488 antibody (1:100) for 1 h. The nuclei were counterstained with 1 μ g/mL DAPI and mounted with Fluoroshield™. The cells probed with HSP90 and HSP70 were photographed under Imager A2 fluorescence microscope (Carl Zeiss, Germany), and the HSF-1 probed cells were photographed using the LSM 800 confocal microscope (Carl Zeiss, Germany) at $\times 400$ magnification.

Immunoblot analysis

Post 24 h of 17-AAG (5 μ M) and TCCP treatment (25, 50, and 100 μ M) of confluent culture of MDA-MB-231 cells in 100-mm petri dishes, the cytosolic and nuclear fractions were prepared following a modified method of Dignam fractionation (Mercier et al. 1999) or whole cell lysate was prepared using 250 μ L ice-cold radioimmunoprecipitation assay (RIPA) buffer to comprehensively study the HSF-1 localization, its phosphorylation status, and its effect on HSP90 and HSP70 expression. Also, the effect of 17-AAG (5 μ M) and TCCP (25 μ M) on activation of upstream kinases such as AKT and ERK was studied at different time intervals (0, 12, and 24 h). Briefly, 80 μ g of protein was fractionated by SDS-PAGE and the proteins were transferred onto PVDF membranes. The membranes were blocked overnight in 5% BSA/TBST at 4 °C, incubated with different primary antibodies diluted in 2% BSA/TBS at room temperature for 2 h. Later,

the membranes were incubated with HRP-conjugated secondary antibodies at room temperature for 1 h. GAPDH was used as an internal loading control for cytosolic and whole cell lysate blots and Lamin B1 for nuclear fraction. Protein bands were visualized using the ECL method (Clarity Western, Bio-Rad) and documented using the G:BOX chemiluminescence imaging system (Syngene, Cambridge UK).

qPCR analysis

Total RNA was extracted using RiboZol reagent (Amresco) from MDA-MB-231 cells 24 h post treatment with TCCP (25, 50, and 100 μ M), 17-AAG (5 μ M), or with vehicle (0.1% DMSO). Complementary DNA (cDNA) was prepared using total RNA (2 μ g) from respective groups along with oligo(dT) primers (K1652, Thermo Scientific). The resulting cDNA was used as template in triplicates for qPCR analysis. Results were normalized with GAPDH control amplification using a comparative Ct method ($2^{-\Delta\Delta C_t}$). Average fold change in gene expression was obtained for each transcript. The sequences of the PCR primer pairs used were as follows:

HSF-1 sense CCATGAAGCATGAGAATGAGGC
 HSF-1 antisense CTTGTTGACGACTTTCTGTTGC
 HSP90 sense CCAGTTTGGTGTCTGGTTTCTAT
 HSP90 antisense CTGGGTATCGTTGTTGTGTTTTG
 HSP70 sense GATGTGTCTGGTTCTCTCCATTG
 HSP70 anti sense CTCCATGAAGTGGTTCACGA

Wound healing assay in MDA-MB-231 cells

The wound healing assay was performed as described previously (Valster et al. 2005). MDA-MB-231 cells were grown to confluency in six-well plates, washed with PBS and serum-starved overnight, and treated with 10 μ g/mL of mitomycin C for 2 h. The monolayer was wounded vertically with a sterile 10- μ L micro-pipette tip in the presence of PBS. The wells were washed gently to remove detached cells. Two milliliters of basal media with VEGF (10 ng/mL), containing TCCP (25 and 50 μ M) or 17-AAG (5 μ M) or vehicle, was added and incubated. The initial wound healing and the movements of cells in the scratched area were photographically monitored at 0, 6, 12, and 24 h post treatment.

Effects of TCCP treatment in vivo

EAT cells or mouse mammary carcinoma cells (5×10^6) were injected intraperitoneally into 10-week-old female Swiss albino mice and fed *ad libitum*. Their growth was recorded every day until the 12th day (Gururaj et al. 2002). These cells grow in the peritoneum forming an ascites tumor with massive abdominal swelling. To verify whether TCCP treatment inhibits

tumor growth and angiogenesis in vivo, intraperitoneal injections of TCCP (50 or 100 μg) for test groups and vehicle (0.1% DMSO in PBS) for the control group were given to three different groups ($n = 6$) of EAT-bearing mice every day after the 6th day of transplantation. The animals were sacrificed on the 13th day; tumor cells along with ascites fluid were harvested from the peritoneal cavity. The pelleted cells were counted by the trypan blue dye exclusion method and also used for immunoblot analysis. The abdominal wall was extended, and the exposed peritoneum was examined for vascularization and photographed.

HSF-1, HSP90, and HSP70 immunohistochemistry

To study the in vivo effect of TCCP treatment on the expression of HSP90, HSP70, and HSF-1 in EAT-bearing mice, microtome sections (5 μm) of paraffin-embedded peritoneum tissue taken from treated and untreated mice were processed for immunohistochemistry as described earlier (Gururaj et al. 2002). Briefly, sections were dewaxed, rehydrated, and blocked for endogenous peroxidase with 3% hydrogen peroxide. Antigen retrieval was done by the heat-induced epitope retrieval method and blocked in serum for 30 min, followed by PBS washing and incubation with anti-HSP90, anti-HSP70, and anti-HSF-1 primary antibodies for 1 h, followed by washing and incubation with HRP-tagged secondary antibody. The SS polymer-HRP detection kit was used for DAB staining (BioGenex, Hyderabad). Subsequently, slides were counter-stained with hematoxylin (2%). After successive dehydration, coverslips were mounted using DPX and evaluated using an Axio Imager A2 bright-field microscope (Carl Zeiss) and photographed ($\times 40$).

Immunohistological analysis by H&E and CD31 staining

To study the effect of TCCP on the reduction of micro-vessel density (MVD), the peritoneum tissues from TCCP-treated, vehicle-treated control, and non-tumor-bearing normal mice were fixed in 10% formalin. Microtome paraffin sections (5 μm) of peritoneum were dewaxed, rehydrated, and stained with hematoxylin and eosin. Coverslips were mounted using DPX, and MVD was determined using the hot spot method in 10 random fields and photographed using an Axio Imager A2 bright-field microscope (Carl Zeiss) and photographed ($\times 40$).

The effect of the TCCP on proliferating endothelial cells was determined by staining the paraffin sections with anti-CD31 antibody as described earlier (Gururaj et al. 2002). Sections were processed using an SS Polymer-HRP Detection Kit (BioGenex, Hyderabad) and probed with anti-CD31 (PECAM-1) antibody, counterstained with 2% hematoxylin, and photographed using an Axio Imager A2 bright-field microscope (Carl Zeiss) and photographed ($\times 40$).

Rat corneal micro-pocket assay

The effect of TCCP on neovascularization in vivo in a non-tumor context was studied by implantation of TCCP along with rVEGF formulated in hydron pellets (Sigma Co., USA) (Nataraj and Salimath 2013) into rat corneas. In brief, pellet was prepared by dissolving hydron polymer in ethanol along with 10 ng VEGF per pellet with and without TCCP (50 and 100 μg /pellet). Female Wistar rats were anesthetized by ketamine (20 mg/kg, IM), and hydron pellets were implanted into the micro-pockets made in the cornea. The cornea was covered with gentamicin drops once a day and photographed on the 7th day using a stereo dissection microscope with a CCD camera ($\times 40$).

Statistical analysis

The data were analyzed in triplicates, and values were expressed as mean $\pm$ SEM using one-way analysis of variance (ANOVA) Dunnett's test. For all tests, $p < 0.01$ (**) was considered statistically extremely significant, significant when $p < 0.05$ (*), and non significant when $p > 0.05$ (ns).

Results

Purification and structural characterization

The butanolic extract from leaves of *T. cordifolia* was purified on pre-loaded silica cartridges. Fractions with similar R_f values on HPTLC were pooled and analyzed for its homogeneity by LC-MS. The pure fraction with a mass of 379 Da (Fig. 1a) was subjected to structural analysis by adopting IR (Fig. 1b) and NMR (Fig. 1c and Supplementary data) techniques. Based on IR spectroscopy, the absorption band in the 1657–1737 cm^{-1} region was attributed to carbonyl and alkene C=C groups and a broad band in the 3400 cm^{-1} region was due to NH moiety of pyrrole. Further, analyzing the 2D [^1H , ^{13}C] HSQC spectrum (Fig. 1c) of the molecule, the probable structure was deduced (Fig. 1d) and was found to be a new molecule from *T. cordifolia*.

Cytotoxicity of TCCP on MDA-MB-231 cells

The relative cytotoxicity was determined by growing the MDA-MB-231 cells in the presence of increasing concentrations of TCCP or 17-AAG for 12-, 24-, and 48-h time intervals. Both TCCP and 17-AAG inhibited cell growth in a time- and concentration-dependent manner, assessed by trypan blue dye exclusion assay with the IC_{50} values of 68.2 and 6.11 μM following 24 h incubation, respectively (Fig. 2a, b).

Fig. 1 Characterization. **a** LS-MS spectrum. **b** Infrared spectrum. **c** 2D $[^{13}\text{C}-^1\text{H}]$ heteronuclear single quantum correlation (HSQC) NMR spectrum. **d** Molecular structure of TCCP

Fig. 2 Cytotoxicity and metabolic response of TCCP or 17-AAG on MDA-MB-231 cells. **a** In vitro cytotoxicity and IC_{50} assessment of TCCP treatments by trypan blue dye exclusion assay. Cells (3×10^4) were treated with either TCCP (10 to 100 $\mu\text{M}/\text{mL}$) or **b** 17-AAG (1 to 20 $\mu\text{M}/\text{mL}$) for 12, 24, and 48 h; washed with PBS; trypsinized stained; and counted in a hemocytometer. Data are presented as the mean $\pm$ SEM

of three independent experiments. **c** Metabolic response to increasing concentrations of TCCP treatments assessed by MTT assay. Cells (3×10^4) were treated with TCCP, 17-AAG, or vehicle for 24 h and incubated with MTT solution, and the formazan crystals dissolved in DMSO and read at 570 nm. Data are presented as the mean $\pm$ SEM of three independent experiments. $**p < 0.01$, $*p < 0.05$

Fig. 3 TCCP suppresses HSP expression and initiates cytosolic translocation of HSF-1. **a** Representative images of cellular localization of HSP90 and HSP70 post TCCP or 17-AAG treatments assessed by immunolocalization studies. Cells (5×10^4) were grown on coverslips, treated with TCCP or 17-AAG or vehicle, and incubated for 24 h and processed as described in the “Material and methods” section and

photographed. **b** Representative images of immunolocalization of HSF-1 transcription factor post TCCP or 17-AAG treatment by confocal microscopy. Indirect immunofluorescent labeling of HSPs and HSF-1 proteins was by Atto 488-tagged secondary antibody and nuclei counterstained using DAPI

Metabolic response of MDA-MB-231 cells to TCCP treatment

The effect of increasing concentrations of TCCP for 24 h incubation on the metabolic response and viability of MDA-MB-231 cells was assessed using MTT assay. The results (Fig. 2c) showed that a similar trend of dose-dependent cytotoxicity compared to that of trypan blue dye exclusion assay showed 50% inhibition at 26.49 μ M, while the 17-AAG-treated cells showed 76.2% inhibition in metabolic response in comparison to vehicle-treated cells. Thus, based on the trypan blue dye exclusion and MTT assay results, we carried out all the in vitro experiments using two concentrations of TCCP (25 and 50 μ M) and 5 μ M of 17-AAG.

TCCP induces intracellular trafficking of HSF-1 and suppresses HSP expression

A high level of HSF-1 nuclear localization in breast cancer is correlated to metastasis and poor prognosis (Mendillo et al. 2012). Since HSF-1 regulates HSP expression, mediated by proteotoxic stress (Santagata et al. 2011a); we studied the effect of TCCP treatment on the localization of HSP90,

HSP70, and HSF-1 in MDA-MB-231 cells using indirect immunofluorescence. The HSP90 and HSP70 proteins (Fig. 3a) which accumulated in the cytoplasm in the vehicle-treated cells appeared to diminish upon TCCP treatment in a dose-dependent manner, with HSP90 diminishing to a greater extent than HSP70. However, there was a drastic decrease in HSP90 and an increased HSP70 accumulation in the cytosol of 17-AAG-treated cells. Additionally, the HSF-1 immunolocalization was found to be mostly concentrated in the nucleus of vehicle-treated cells (Fig. 3b) which, upon TCCP treatment, relocated to the cytoplasm at higher doses. But the 17-AAG treatment did not appear to affect the localization of HSF-1 that remained in the nucleus.

TCCP inactivates HSF-1 and modulates HSPs, AKT, and ERK activation

Following the leads from the immunolocalization results, we studied the influence of TCCP treatment on the protein expression profile of HSF-1 along with HSP90 and HSP70 by western blot analysis to ascertain the levels of HSF-1 in both the cytosolic and the nuclear fractions. Treatment with 25 μ M TCCP augmented the cytosolic localization of HSF-1 that was

Fig. 4 TCCP downregulates HSP90 and HSP70 through repression of HSF-1 activation. Dose response of TCCP induced repression of the HSF-1 transcription factor assessed by immunoblot analysis. **a** Cytosolic protein fraction. **b** Nuclear protein fraction. Confluent MDA-MB-231 cells in 100-mm petri dishes were treated with 17-AAG, vehicle, or TCCP for 24 h. The cytosolic and nuclear fractions were prepared following a modified method of Dignam fractionation, and clarified lysates (80 µg) were electrophoresed on 12% SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% BSA/TBS, membranes were probed with anti-HSF-1 or anti-pHSF-1³⁰⁷

separately for cytosolic and nuclear fractions. **c** 17-AAG and TCCP induced modulation of HSP90 and HSP70 expression and **d** AKT and **e** ERK phosphorylation at the indicated time points assessed by immunoblotting using whole cell RIPA buffer lysates. HRP-tagged secondary antibody was used for probing. GAPDH was used as an internal loading control for cytosolic or whole cell lysate blots and Lamin B1 for nuclear fraction blots and developed using the ECL method. Data are presented as the mean $\pm$ SEM of three independent experiments; ** $p < 0.01$, * $p < 0.05$, and ns is $p > 0.05$

further bolstered with 50 and 100 µM concentrations in a dose-dependent manner after 24 h (Fig. 4a). In contrast, the nuclear fraction showed a decrease in the HSF-1 levels (Fig. 4b). In support of these observations, we looked into the phosphorylation status of HSF-1 at Serine³⁰⁷, one of the residues known to negatively regulate HSF-1 transcriptional activity (Guettouche et al. 2005). As predicted, post 24 h TCCP treatment, HSF-1 exhibited a dose-dependent increase in the phosphorylation on the Ser³⁰⁷ residue in both cytosolic (Fig. 4a) and nuclear fractions (Fig. 4b).

On the other hand, since HSP expression is regulated by HSF-1 and owing to phosphorylation of HSF-1 on Ser³⁰⁷ by TCCP, we examined the time kinetics of TCCP treatment (25 µM) on protein accumulation of HSP90 and HSP70 using the whole cell lysate and found a significant downregulation of HSP90 and a moderate downregulation of HSP 70 in a time-dependent manner (Fig. 4c). Furthermore, we also verified the interplay of AKT or ERK1/2 activities during TCCP treatment in phosphorylating HSF-1. Immunoblot (Fig. 4d) results revealed that TCCP treatment significantly diminished

the phosphorylation of AKT on the Ser⁴⁷³ residue which is known to be associated with kinase activity. Additionally, TCCP induced hyperphosphorylation of ERK1/2 in a time-dependent manner, a kinase responsible for phosphorylating HSF-1 on Ser³⁰⁷.

Unaffected HSF-1 gene expression and suppressed HSP90 and HSP70 gene expression

Quantitative PCR analysis to study the gene expression profiles of *HSF-1* and its dependent *HSP90* and *HSP70* genes was performed using the total RNA isolated from vehicle-treated, 17-AAG-treated, and TCCP-treated MDA-MB-231 cells. The results normalized to GAPDH expression showed no change or negligible decrease in HSF-1 transcripts by 0.1-, 0.1-, and 0.3-fold with TCCP treatment and a 0.01-fold decrease after 17-AAG treatment, relative to the vehicle-treated control after 24 h (Fig. 5a). Nevertheless, HSP90 gene expression decreased in a dose-dependent manner by 0.22-, 0.6-, and 0.75-fold upon TCCP treatment, and inconsequentially

Fig. 5 Relative mRNA transcript levels of HSF-1, HSP90, and HSP70, post TCCP or 17-AAG treatments assessed by qPCR. **a** TCCP or 17-AAG treatments have negligible influence on gene expression of HSF-1. **b** HSP90 gene expression is significantly decreased by TCCP but unaltered by 17-AAG. **c** HSP70 gene expression is marginally decreased by TCCP but drastically increased by 17-AAG. Total RNA was isolated

from vehicle-, 17-AAG-, and TCCP-treated cells, and equal amounts of RNA were converted into cDNA and the mRNA levels of HSF-1, HSP90, and HSP70 were quantified by qPCR and normalized to GAPDH expression. Data are presented as the mean $\pm$ SEM of three independent experiments. * p < 0.05 significant and ** p < 0.01 significantly different from the control

decreased 0.04-fold after 17-AAG treatment (Fig. 5b). On the other hand, HSP70 gene expression after TCCP treatment decreased slightly by 0.1-, 0.2-, and 0.3-fold but significantly increased by 1.8-fold after 17-AAG treatment (Fig. 5c), corroborating the earlier observations of HSF-1, HSP90, and HSP70 protein downregulation as evidenced in immunolocalization and immunoblot studies.

TCCP inhibits VEGF-induced MDA-MB-231 cell migration

Since anti-HSP drugs are also anti-angiogenic in nature, we evaluated the same using TCCP. The inhibition of wound closure of MDA-MB-231 cells by TCCP clearly showed decreased cell migration in a dose-dependent manner, whereas the 17-AAG-treated cells moderately closed the wound, while the control cells in the presence of VEGF rapidly closed the wound (Fig. 6a). Quantitative analysis of wound healing assay (Fig. 6b) showed that less number of cells were involved in closure of the wound in TCCP (50 μ M) treatment, compared with that of 17-AAG or VEGF treatments.

Effect of in vivo TCCP treatment on HSF-1, HSP90, and HSP70 in Ehrlich ascites tumor mouse model

HSF-1 is involved in tumorigenesis in mouse models (Dai et al. 2007; Meng et al. 2011; Min et al. 2007). Accordingly, the transplantable undifferentiated Ehrlich ascites tumor (EAT) mouse mammary carcinoma model was used to study the effect of TCCP treatment on EAT cell proliferation, ascites secretion, angiogenesis, and expression of HSF-1, HSP90, and HSP70 proteins in vivo. There was a considerable decrease in body weight (Fig. 7a), secreted ascites volume (Fig. 7b), and EAT cell number (Fig. 7c) in a dose-dependent manner compared to the vehicle-treated control.

Also, there was a concomitant dose-dependent downregulation in the expression of HSF-1, HSP90, and HSP70 upon TCCP treatment compared to the control group evidenced by western blot analysis (Fig. 7d). Further, the immunohistochemistry of peritoneum tissue sections revealed visibly stronger immuno-staining with HSF-1 antibody in vehicle-treated control peritoneum, which was significantly less upon treatment with TCCP (100 μ g) while the untreated normal peritoneum showed weak staining (Fig. 7e). The same trend was also seen in peritoneum sections probed with anti-HSP90 and HSP70 antibodies. Hence, the purified compound unequivocally proved to be a potential molecule in inhibiting heat shock response as validated by both in vitro and in vivo studies.

TCCP inhibits peritoneal angiogenesis in EAT mouse model

Based on previous reports, HSF-1 is known to support tumor angiogenesis, which has been studied in *HSF-1* knockout mice and xenograft models (Vydra et al. 2014). As angiogenesis is evident in the inner peritoneal lining of EAT-bearing mice, it is a reliable model for studying in vivo angiogenesis. A decrease in peritoneal angiogenesis was evident upon TCCP treatment compared to EAT-bearing, control mice (Fig. 8). Not all vessels in the peritoneum were affected by the treatment with TCCP except the newly formed capillaries. The angioinhibitory effect was further verified by histological examination of the peritoneum sections using H&E and CD31 immunostaining to study the microvessel density (MVD). The results revealed that TCCP treatment significantly suppressed neo-angiogenesis in the peritoneum of tumor-bearing mice (Fig. 8), supported by the drastic decline in MVD (36 and 68%) in the treated group compared to the untreated group. Likewise, immunohistological analysis for the CD31 marker

Fig. 6 Suppression of cell migration by TCCP assessed by wound healing assay. **a** Confluent MDA-MB-231 cells in a six-well plate were serum starved overnight and treated with mitomycin c, and a scratch was made on the cell monolayer. Cell debris was washed, and the cells were cultured in basal medium containing VEGF along with indicated concentration of TCCP, 17-AAG, or vehicle. The wound closure was photographed at indicated time intervals. Images are representative of at least 10 independent locations that were monitored over the length of each wound. **b** Quantification of the cells involved in wound closure. * $p < 0.01$ = non-significant and ** $p < 0.01$ considered significantly different from the control. Values are expressed as mean $\pm$ SEM of three replicate analyses

present in proliferating endothelial cells revealed the reduction in the number of proliferating endothelial cells in the treated group in comparison to untreated EAT-bearing mice or the normal mouse.

Neovasculture regression in rat cornea assay

The rat cornea micro-pocket assay is commonly used as an in vivo validation assay for studying the angioinhibitory activity of antiangiogenic molecules. As revealed by the data (Fig. 9), TCCP was found to effectively decrease VEGF-induced angiogenesis by lowering the length of the capillaries

by 25 and 60% as well as the area of neovasculture by 31 and 61% upon TCCP treatment with 50 and 100 μ g, respectively, in comparison to extensive angiogenesis induced by VEGF. Thus, by considering our collective results, we presume that downregulating HSF-1 could also diminish peritoneal and VEGF-mediated angiogenesis.

Discussion

This is a maiden report of a bioactive pyrrole derivative (TCCP), isolated from the leaves of *T. cordifolia* illustrating

Fig. 7 TCCP inhibits EAT cell proliferation and downregulates HSF-1, HSP90, and HSP70 in vivo. **a** Reduction in the body weight of EAT-bearing mice. **b** Cell number and **c** secreted ascites volume in untreated and treated groups. EAT-bearing mice were injected (i.p) with TCCP or vehicle from the 6th day of transplantation till the 12th day. The mice were sacrificed, cells were pelleted and counted, and volume of ascites was noted. **d** Immunoblot analysis and quantification of HSF-1, HSP90, and HSP70 protein expression in control and TCCP-treated EAT cells; cells were harvested from mice post treatment, and whole cell RIPA buffer lysates were prepared and immunoblotted for HSF-1, HSP90,

and HSP70 proteins. GAPDH was used as an internal loading control. **e** Representative photographs of immunohistochemical staining for HSF-1, HSP90, and HSP70 proteins in the peritoneal section of tumor-bearing control, TCCP-treated, and normal mice. Post treatment, mice were sacrificed and the peritoneum was dissected and 5- μ m microtome sections were stained using IHC methodology for HSF-1, HSP90, and HSP70 using respective antibodies and HRP-tagged secondary antibody and photographed using a bright-field microscope. Values are expressed as mean $\pm$ SEM of three replicate analyses; ** $p < 0.01$, * $p < 0.05$, and ns is $p > 0.05$

Fig. 8 TCCP mediated inhibition of peritoneal angiogenesis in vivo. Representative photographs of vascularization changes in the peritoneal wall upon TCCP treatment. Peritoneum sections of normal and EAT-bearing mice with indicated treatments. Lane 1—normal mouse (non-tumor-bearing), lane 2—control (tumor-bearing) treated with vehicle (0.1% DMSO in PBS), lane 3—tumor-bearing, treated with 50 µg TCCP, and lane 4—tumor-bearing, treated with 100 µg TCCP. The number of newly formed capillaries was assessed by the peritoneum

pictures. MVD assessed by hematoxylin and eosin staining of peritoneum sections. CD31-positive staining was assessed by PECAM-1 immunohistochemical staining of peritoneum sections. Arrows indicate the presence of endothelial cell lining and infiltrating tumor cells in the peritoneum of the mouse. Values are expressed as mean $\pm$ SEM of three replicate analyses. * $p < 0.05$ significant and ** $p < 0.01$ significantly different from the control

significant repression of tumor cell proliferation, tumor angiogenesis, and inhibition of HSPs via inactivation of HSF-1. The molecular mechanism of action of TCCP has been delineated. The molecule was shown to have specific targets such as HSF-1, AKT, and ERK1/2 in order to bring about its anti-tumor activity using HSP90 as a major canonical downstream player.

Pyrrole is a prominent biologically active scaffold displaying a plethora of bioactive properties. The pyrrole ring system comprising different pharmacophores has expedited pharmaceutical research with numerous bioactive compounds. Natural products are a good source of analogs containing pyrrole rings with diverse properties. Pharmaceuticals containing pyrrole ring structures possess potent anti-cancer, antibacterial, anti-fungal, anti-malarial, anti-psychotic, anxiolytic, and many more pharmacologically important actions (Bhardwaj et al. 2015). More research is needed on such pyrrole-based natural compounds to tap into its therapeutic potential. With reference to anti-cancer agents, several pyrrole-based natural compounds such as roseophilin 226, prodigiosin 227, and synthetic aryl pyrroles like LB42908 have excellent anti-tumor activity (Bhardwaj et al. 2015).

Highly malignant breast cancer and several human tumors overexpress HSPs. A high level of HSPs is associated with tumor

cell survival as well as poor prognosis. Hence, downregulation of HSPs, especially HSP90 and HSP70, is a sensible strategy for cancer therapy especially in cancers with restricted therapeutic options. Furthermore, the transcription factor HSF-1, by itself, is a robust and versatile regulator of cancer. Primarily, HSF-1 upregulates the expression of HSPs that protect the oncoproteins required for tumorigenesis and survival, from degradation, such as mutant p53 proteins. HSF-1 regulates glycolysis and lipid metabolism and regulates different signaling pathways, such as HuR-HIF-1, Slug, PKC, NF- κ B, PI3K-AKT-mTOR, VEGF, and MAPK pathways that are essential for cell proliferation, anti-apoptosis, tumor angiogenesis, EMT, migration, invasion, and metastasis. Also, HSF-1 regulates the expression of both miRNAs and lncRNAs (Jiang et al. 2015; Santagata et al. 2011a). The significance of HSF-1 in carcinogenesis has been established by the reduced tumor formation in *Hsf1* knockout mice. HSF-1 promotes the growth of human tumor cells in culture since reduced HSF1 levels significantly reduce their proliferation and survival (Mendillo et al. 2012). The heat shock response (HSR) elicited by HSF-1 in cancer cells involves transcription of genes that are not induced by heat shock (Mendillo et al. 2012), leading to metastasis and death in cancers of breast, colon, and lung (Scott et al. 2011).

Fig. 9 The corneal neovascularization inhibited by TCCP. Representative photographs of neovascularization observed in rat corneas: hydon polymer with vehicle (0.1% DMSO in PBS) negative control, hydon polymer + VEGF (10 ng) positive control, and hydon polymer + VEGF + TCCP (50 or 100 µg) were tests. Histograms showing length of blood vessels and vascular area in control and treated corneas both assessed by ImageJ

software. Details of the experiments are described in the “[Material and methods](#)” section. After 7 days of incubation, the corneas were photographed at $\times 40$ magnification through a stereo zoom microscope. Values are expressed as mean $\pm$ SEM of three replicate analyses. * $p < 0.05$ significant and ** $p < 0.01$ considered extremely significantly different from the VEGF-treated positive control

In the present study, we have used MDA-MB-231 cancer cells, reported to be a highly metastatic, triple negative breast cancer cell line (TNBC). The strategy of inhibition of HSPs through HSF-1 as a treatment modality in TNBC has not been reported widely. Previously, a CRISPR/Cas9 gene knockout study with Hsp90 α in HIF-1 α -overexpressing MDA-MB-231 cells has shown that overexpression of HSP90 α is vital to counteract the hypoxia-triggered cell death and to cope with the hostile hypoxic tumor environment (Dong et al. 2016). Also, targeting the heat shock response or HSF-1 using small molecules sensitizes tumor cells to HSP90 inhibition (Schilling et al. 2015). The experimental data with our small molecule, TCCP, on cell proliferation studies clearly revealed that TCCP inhibits cell proliferation and facilitates inactivation and translocation of HSF-1 from the nucleus to the cytosol. However, this trend was not seen in 17-AAG-treated cells. HSF-1 is normally found to shuttle between the nucleus and the cytoplasm, but when activated by stressors, it accumulates within the nucleus as was seen in vehicle-treated cells. On the contrary, based on the notion derived from the immunolocalization results that HSF-1 may be influencing the expression of HSPs, we investigated the accumulation of HSF-1 and its phosphorylation status on the Serine³⁰⁷ residue in the cytosolic and nuclear fractions. Our data indicates that TCCP phosphorylates HSF-1 on Ser³⁰⁷, similar to

an earlier report (De Bock et al. 2011). Further, accumulation of HSF-1 in the cytoplasm and reduction in the nucleus, post TCCP treatment, may be explained based on a previous study which shows that phosphorylation on the two conserved serine residues, Ser³⁰⁷ and Ser³⁰³, on HSF-1 is essential for its association with 14-3-3 ϵ , leading to the repression of HSP genes due to sequestration of HSF-1 into the cytoplasm away from the promoters of *hsp* genes. Similar results have been previously reported with localization of HSF-1 after wortmannin exposure resulting in increased cytosolic localization and decreased levels of nuclear HSF-1 after 24 h incubation in K562 cells (Mustafi et al. 2010). Additionally, the Ser³⁰⁷-phosphorylated HSF-1 localization was unaltered, wherein it was found to be increased in the cytosolic and nuclear fractions in a dose-dependent manner. The results compel us to arrive at a mechanism whereby phosphorylation of HSF-1 on Ser³⁰⁷ consequently downregulates the expression of the HSPs. Also, we have verified whether HSF-1 through a cross talk with AKT and ERK mediates downregulation of HSP90 and HSP70 expressions. Our results clearly show that TCCP is able to regulate AKT activity by dephosphorylation of AKT at Ser⁴³⁷ and phosphorylation of ERK1/2. Thus, it is clear that TCCP abrogates expression and nuclear translocation of HSF-1 and thus downregulates the HSP expression via AKT inactivation, which is sustained further by the

phosphorylation of ERK1/2, the kinase responsible for inactivation of HSF-1. Therefore, one can effectively inhibit HSP gene expression by concerted action of both AKT and ERK1/2 on HSF-1. Similar results of blockade of AKT phosphorylation by the PI3K inhibitor wortmannin revealed that HSP70 expression is dependent on activated AKT (Mustafi et al. 2010).

Our data on gene expression analysis using qPCR reveals that TCCP is able to decrease the transcription of HSP90 by a considerable extent compared to that of HSP70. However, TCCP treatment had very little effect on the levels of HSF-1 transcripts. On the other hand, 17-AAG drastically increased the HSP70 transcripts and did not alter either HSP90 or HSF-1 transcription. Furthermore, TCCP inhibited the migration of cells in a dose-dependent manner as seen in the wound healing assay. This might be explained by the fact that HSF-1 regulates the migration and EMT pathway, and HSF-1 has been identified as one among the six potent tumor metastasis-promoting genes (Scott et al. 2011).

Further, from our in vivo EAT mouse model studies, TCCP significantly suppressed HSP expression in the tumor-bearing mouse as observed by immunohistochemistry and western blot analysis. TCCP also significantly inhibited EAT tumor growth and suppressed tumor angiogenesis. Inhibition of HSF-1 strongly inhibits secretion of vascular endothelial growth factor (VEGF), the major growth factor responsible for neovascularization, as

well as other targets such as HIF-1 α , CAIX, and Glut-1 (Gabai et al. 2012). An angiogenesis-regulating mechanism mediated by HSPs has been reported, wherein the HSP90-HSP70 complex is involved in targeting the VEGFR2 receptor for degradation. Geldanamycin blocks VEGF-A signaling. Inhibition of HSP90 results in degradation of VEGFR2 by HSP70 by the endosome-lysosome degradation pathway (Bruns et al. 2012). Comprehensively, the net result of TCCP action precipitates down to the inhibition of tumor-induced angiogenesis in an ascites tumor model, where we have shown that TCCP inhibits tumor cell proliferation, ascites secretion, and peritoneal angiogenesis as is validated by an endothelial cell proliferation marker, CD31 staining and MVD scoring. Further, TCCP effectively inhibited VEGF-induced neovascularization even in a non-tumor context. Our data on inhibition of angiogenesis in rat corneal micro-pocket assay in the presence of VEGF further substantiates the anti-angiogenic activity of TCCP.

Thus, the present study provides direct evidence that TCCP, isolated from *T. cordifolia*, has potent anti-HSP, anti-angiogenic, and anti-tumor activities mediated through suppression of HSF-1 and AKT and activation of ERK.

In summary, TCCP isolated from the leaves of *T. cordifolia* can further be developed for therapeutic purpose. The molecular mechanism of TCCP action which has been elucidated in this work (Fig. 10) clearly shows that phosphorylation of

Fig. 10 The graphical representation of mode of action of TCCP-induced HSP downregulation in MDA-MB-231 cells

target HSF-1 via ERK1/2 and dephosphorylation of AKT-1 modulate the expression of HSPs in order to inhibit tumor growth, angiogenesis, and migration. TCCP with potential anti-HSP effects can be a potential biomolecule for clinical exploitation as cancer therapeutics either alone or as combinatorial therapy with other known HSP inhibitors.

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