Heat shock protein 27 is a potential indicator for response to 
YangZheng XiaoJi and chemotherapy agents in cancer cells

SIONED OWEN1,2*, HUISHAN ZHAO1,3*, ALWYN DART1,2, YAMEI WANG1,3,
FIONA RUGE1,2, YONG GAO4, CONG WEI4,5, YILING WU4,6* and WEN G. JIANG1,2

1Cardiff China Medical Research Collaborative, Cardiff University School of Medicine; 2Cardiff University-Capital Medical 
University Joint Centre for Biomedical Research, Cardiff University, Cardiff, UK; 3Cancer Institute and Key Laboratory 
of Invasion and Metastasis (Beijing), Capital Medical University, Xitoutiao, Fengtai, Beijing; 4Yiling Medical 
Research Institute, Shijiazhuang; 5State Key Laboratory of Collateral Disease Research and Innovation Medicine, 
Shijiazhuang; 6Key Disciplines of State Administration of TCM for Collateral Disease, Shijiazhuang, Hebei, P.R. China

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Abstract. Heat shock protein 27 (HSP27) is a member of the 
heat shock protein family which has been linked to tumour 
progression and, most interestingly, to chemotherapy resis-
tance in cancer patients. The present study examined the 
potential interplay between HSP27 and YangZheng XiaoJi, a 
traditional Chinese medicine used in cancer treatment. A range 
of cell lines from different tumour types including pancre-
atic, lung, gastric, colorectal, breast, prostate and ovarian 
cancer (both wild-type and resistant) were used. Levels and 
activation of HSP27 and its potential associated signalling 
pathways were evaluated by protein array and western blot-
ing. Knockdown of HSP27 in cancer cells was achieved using 
siRNA. Localisation and co-localisation of HSP27 and other 
proteins were carried out by immunofluorescence. Cell growth 
and migration were evaluated in their response to a range of 
chemotherapeutic agents. The present study first identified, by 
way of protein array, that YangZheng XiaoJi was able to inhibit 
the phosphorylation of HSP27 protein in cancer cells. We 
study also demonstrated that YangZheng XiaoJi was able to 
sensitise cancer cells including those cells that were resistant to 
chemotherapy, to chemotherapeutic agents. Finally, knocking 
down HSP27 markedly reduced the migration of cancer cells 
and increased the sensitivity of cancer cells to the inhibitory 
effect on cellular migration by YangZheng XiaoJi. YangZheng 
XiaoJi can act as an agent in first sensitising cancer cells to 
chemotherapy and secondly to overcome, to some degree, 
chemoresistance when used in an appropriate fashion in 
patients who have active HSP27.

Introduction

The heat shock proteins (HSPs) are ubiquitously expressed in 
a almost all cells and across species. The protein family was so 
named owing to their initial discovery, namely, identified from 
cells under heat shock and is now known to be involved in a 
number of other cellular stresses including heat, carcinogens, 
mechanical and chemical stress (1,2). These proteins serve as 
molecular chaperones and act as a mechanism to assist protein 
folding, repair damaged proteins, or assist in degrading the 
unwanted proteins after stress and injury, thus by doing so 
the proteins protect the injured cells from potentially lethal 
damage (3,4). The HSP proteins are thus important regulators 
in normal physiology, but are also involved in diseases 
including cardiovascular, wound healing and cancer.

Similarly discovered from cells under heat shock, HSP27 
has been found to be widely involved in other types of cell 
stress, including oxidative response (5-8). The HSP27 is a 
protein of approximately 27 kDa in size and belongs to the 
HSP family in which sizes vary between 8-150 kDa. The 
main cellular function of HSP27 is protecting cells from 
becoming apoptotic, or anti-apoptotic. Although this role is 
critically important in homeostasis, it has serious implica-
tions in clinical cancer. Firstly, HSP27 has been found to be 
overexpressed in a variety of human cancers and linked to a 
poor outcome for those cancer patients (9-13); secondly, high 
levels of HSP27 in cancer cells have been shown to promote 
cancer cell growth (14-16); and thirdly, HSP27 was found to 
be extremely elevated following chemotherapy, most probably 
as a protective mechanism to chemical stress (17-19). In the 
latter case, HSP27 and HSP70 were two of the gene products 
that were upregulated in a cisplatin resistant ovarian cancer 
cell line (19). Thus, this has caused an enormous challenge

Correspondence to: Professor Wen G. Jiang, Cardiff China 
Medical Research Collaborative, Cardiff University School of 
Medicine, Henry Wellcome Building, Cardiff CF14 4XN, UK 
E-mail: jiangw@cf.ac.uk

*Contributed equally

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caspases, Focal adhesion kinase, lung cancer, pancreatic cancer, 
ovarian cancer, cellular migration
to cancer treatment and is frequently linked to chemotherapy resistance in cancer.

The key mode of activation of HSP27 is by phosphorylation. Phosphorylation of HSP27 allows itself to form oligomers which may facilitate the chaperone process under cellular stress (20,21). Phospho-HSP27 interacts with other pro-apoptotic proteins such as DAXX and ASK1 and therefore by blocking pro-apoptotic protein interaction blocks the apoptotic pathway (22,23). Other known pathways of HSP27 include interaction with AKT1 and the oestrogen receptor. By interacting with these signalling proteins, phospo-HSP27 blocks some of their actions in cancer cells. The most commonly reported sites of phosphorylation with HSP27 are S15, S78, S82 and S86. Consequently, phosphorylated HSP27 protein levels have been found to be frequently raised in clinical cancer tissues and chemoresistant cancer cells (24,25).

Together, it is suggested that HSP27 is a valid target for cancer treatment. Methods to either reduce the protein levels of HSP27 or the phosphorylation of HSP27 would be desirable in devising cancer treatment (26). A new anti-HSP27 agent, known as OGX427 (Apatersen) has entered into a late phase clinical trial in patients with various malignant conditions including pancreatic, lung, bladder or prostate cancer, alone or in combination with other chemotherapies (27-30). Early studies have shown a favourable outcome from the new treatment.

There have been clinical trials which have shown that YangZheng XiaoJi (YZXJ), a combination of traditional Chinese medicinal herbs benefit cancer patients; however, how this mechanism of action is achieved remains unknown (31-34). The present study reports an accidental and surprising discovery that a traditional Chinese herbal medicine, known as YangZheng XiaoJi (YZXJ) used in treating patients with cancer, can suppress the phosphorylation of HSP27 and cell functions related to this protein.

Materials and methods

Materials. Antibodies to human HSP27 (sc-13132), caspase-3 (sc-7148), caspase-8 (sc-70501), caspase-9 (sc-17784), phospho-FAK (sc-81493), and GAPDH (sc-32233) were purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA, USA). Therapeutic agents including cisplatin, topotecan, pacilitaxol and 5-FU were purchased from Sigma-Aldrich (Poole, Dorset, UK). Antibodies to FAK (ab131435) and phospho-HSP27 (S86) (ab17938) were purchased from Abcam (Cambridge, UK). FITC/TRITC-conjugated Phalloidin and FITC- and TRITC-conjugated secondary antibodies (fluorescence- and HRT-conjugated) were also from Sigma-Aldrich. Anti-HSP27 siRNA, control siRNA and transfection reagents were also obtained from Santa Cruz Biotechnologies Inc.

Cells. Human gastric cancer (AGS and HGC27), pancreatic cancer (PANC1), ovarian cancer (SKOV3 and COV504), lung cancer (A549 and SKMES1), breast cancer (MDA MB-231), prostate cancer (PC-3) cells were purchased from LGC Standard/ATCC (Southampton, UK). Ovarian cancer cells A2780 and its cisplatin resistant strain A2780/CP70 were gifts from Imperial College London (Dr Euan Stronach).

YangZheng XiaoJi extracts. An extract from YZXJ, named DME25 was prepared using a DMSO based method that has been described in full (35,36). The herbal medicinal formula, YangZheng XiaoJi was obtained from Yiling Pharmaceuticals (Shijiazhuang, HeBei, China). The formula contained the following 16 ingredients: Panax ginseng C.A. Mey, Astragalus membraneus (Fisch.) Bge.var. mongholicus (Bge.) Hsiao, Ligustrum lucidum Ait, Curcuma phaeocaulis Val, Ganodema lucidum, Gynostemma pentaphylla (Thunb) Mak, Atractyloides macrocephala Koidz., Scutellaria barbata D.Don, Oldenlandia diffusa (willd.) Roxb, Poria cocos, Duchesnea indica Focke, Solanum lyratum Thunb, Artemisia scoparia (Bge.) Ki, Cynanchum paniculatum Kitag, Eupolyphaga sinensis Walker, and Gallus domesticus Brisson.

For experimental use, the extract was diluted in the respective cell culture media and the dilution range was between 1:100 to 1:2,000.

Immunofluorescent staining. Cells, plated with test agents in 8-well chamber slides, were first fixed using 4% formalin, lightly permeabilised with 0.1% Triton X100 for 5 min and blocked with a Tris buffer (25 mM, pH 8.4) that contained 7.5% pre-immuned goat serum for 1 h. Primary antibodies (including anti-HSP27, anti-phospho-HSP27 (S86), anti-caspases), diluted in the blocking buffer was added to the respective slides which were kept in the dark at full humidity on a slow moving platform for 1 h, and then washed thoroughly. FITC-tagged secondary antibodies were then added. After 1 h, the slides were washed thoroughly and mounted using FluorSave™ (Calbiochem, Nottingham, UK). TRITC-conjugated phalloidin was diluted at final concentration of 10 µg/ml and added to the cells together with the secondary antibodies. The slides were examined on an Olympus microscope and photographed using a Hamamatsu digital camera. The staining intensity was determined using ImageJ software.

Knockdown of HSP27. Cells at approximate 70% confluence were rinsed with BSS buffer. Anti-HSP27 siRNA, diluted in RNA free water was first mixed with a transfection reagent and left at room temperature for 30 min before being added to the cells. After 7 h medium was replaced and cells were left to incubate for another 24 h. Expression of HSP27 was tested using RT-PCR.

Cell growth assay. Cancer cells were first seeded in 96-well tissue culture plates and allowed to adhere. The test materials and their combinations were then added to the cells, which were subsequently incubated for a period of 72 h. After removing the culture media, cells were fixed with 4% formalin and stained with 0.5% Crystal violet. After extensive washing, the staining was extracted with 10% acetic acid and plates were read on a multiple channel plate reader at 540 nm (Elx800; Bio-Tek, Swindon, UK). The test regimens included chemotherapeutic agent alone, DME25 alone, DME25 and chemotherapeutic agent combination and DME25 pre-treatment followed by chemotherapeutic agent.

Protein arrays for detecting phosphorylation changes. Cancer cells at 90% confluence in two T75 tissue culture flasks were washed with BSS buffer and then placed into a fresh batch
of DMEM supplemented with 5% FCS. After 5 h, treatment was added, again in 5% FCS. After a 5-h period, cells were removed from the flasks with a cell scraper. The cells were pelleted using a centrifuge at 2,500 rpm for 5 min. Lysis buffer was added to the cell pellets and placed on a spinning wheel for 1 h at 4˚C. The lysates were then spun at 12,000 x g for 10 min at 4˚C. The supernatants were carefully collected and the insolubles discarded. Based on absorbance the protein concentration in the cell lysates were quantified using the scatter line chart of microsoft Excel and then adjusted to 2 mg/ml. The samples were stored at -20˚C until use. Antibody based protein arrays, namely KAm850, which has 854 capture antibodies spotted on to each array slides (Kinexus Bioinformatics Ltd., Vancouver, Canada) were used in the present study. The following are the key parameters collected and used for the data analysis: Globally Normalized Signal Intensity, Background corrected intensity values are globally normalized. The Globally Normalized Signal Intensity was calculated by summing the intensities of all the net signal median values for a sample. Z Scores, Z score transformation corrects data internally within a single sample. Z Score Difference, The difference between the observed protein Z scores in samples in comparison. Z Ratios, Divide the Z Score Differences by the SD of all the differences for the comparison.

**Electric cell-substrate impedance sensing (ECIS) based analyses on cell adhesion and cell migration.** Briefly, 96-well W96E1 microarrays were used on the ECIS Ztheta instrument (Applied Biophysics Ltd., Troy, NJ, USA). Lung cancer cells were added to the wells of the array, followed by immediate tracking of cell adhesion over a range of frequencies (1,000 to 64,000 Hz) using automated modules. The adhesion was analysed using the mathematical modelling methods as previously described. For cellular migration, confluent lung cancer monolayers in the arrays were electrically wounded (2,000 mA for 20 sec each), after which the migration of the cells was immediately tracked, again over a range of frequencies. All the experiments were conducted in triplicate.

**Western blotting analysis of HSP27 proteins.** Proteins, prepared in a similar fashion to that for protein analysis, were separated on a SDS-PAGE genes, following transfer of
proteins to nitrocellulose membranes, the membranes were
probed with the respective antibodies in a set protocol that
including extensive washings in between the antibody probing
and finally detecting the antibody signals using chemillumi-
nescence reagents. The membranes were visualised using an
imager (G-Box; Syngene, Cambridge, UK).

**Results**

*DM25 has a marked effect on the phosphorylation of HSP27.*

Using a protein array, we found that the *YZXJ* extract DM25 resulted in a marked reduction of phosphorylation of HSP27,

particularly on Serine86 (S86) phosphorylation, in SKMES-1 lung cancer and PANC-1 pancreatic cancer cells (Fig. 1A and B, respectively).

Localisation of HSP27 and phospho-HSP27 (S86) in cancer
cells, assessed by immunofluorescence. HSP27 was seen
broadly in the nucleus and in cytoplasmic region of lung cancer
cells (Fig. 2A, top panel). It is very interesting to note that
both total HSP27 and phospho-HSP27 was localised in focal
adhesion and pseudopodia regions of the cells. Treatment of
the lung cancer cells, SKMES1 with DM25 resulted in loss
of phospho-HSP27 from the focal adhesion and pseudopodia
regions of the cells, although the changes of total HS P27 did not appear to differ (Fig. 2). The same changes of phospho-HSP27 were seen in pancreatic cancer cells, PANC1 (Fig. 2A, bottom panel) as well as in ovarian cancer cell SKOV3 and gastric cancer AGS cells (data not shown). The inhibition on levels and activation of HSP27 was also demonstrated by western blotting analysis in both the lung and pancreatic cells (Fig. 2B and C, respectively).

Activated HSP27 is co-localised with caspase-9 in cancer cells, which is prevented when cells are treated with DME25. When co-stained for phospho-HSP27 (S86) and caspase-9, it was found that both molecules co-localised in regions of focal adhesion (FAC) and pseudopodia (Fig. 3A and B). It was very interesting to note that when cells were treated with DME25, this pattern of co-localisation appears to break (Fig. 3B).

Phospho-HSP27 and its co-localisation with filamentous actin and caspase-9 in cancer cells and in chemoresistant cancer cells. Using dual fluorescence staining, we have shown that phospho-HSP27 and F-actin did co-localise in FAC regions in both wild-type ovarian cancer A2780 (Fig. 4A, top panels) and in cisplatin resistant A2780-CP70 cells (Fig. 4 bottom panels). The same was seen with lung cancer cells (Fig. 5). In all the cells tested, treatment with DME25 reduced the location of phospho-HSP27 to the FAC region. The co-localisation...
Effect of DME25 on the growth of ovarian cancer cells. A2780/CP70 showed approximately 8 times less sensitivity to cisplatin compared with the wild-type parental cells (Fig. 6). Co-culture of both cisplatin and DME25 resulted in the cells becoming more sensitive to cisplatin, as seen for lung, gastric, pancreatic and ovarian cancer cells (Tables I-V). However, knocking down HSP27 by siRNA did not significantly influence the sensitivity of the cells to the chemotherapeutic agents (data not shown).

HSP27 shows marked influence on cell migration and acts synergistically with YangZheng XiaoJi on cancer cell migration. In light of the co-localisation of HSP27 and phalloidin at the focal adhesion regions of cancer cells and our previous reports on the impact of DME25 on the migration of various cells including cancer cells, we tested how HSP27 might affect cell migration. It was readily demonstrable that knocking down HSP27 resulted in a sustained reduction of cell migration (Fig. 7A and B). It was further confirmed that knocking down HSP27 increased sensitivity of cancer cells to low dosage chemotherapeutic agents and in particular when DME25 was present (Fig. 7C and D).

**Discussion**

The current study stemmed from a surprising finding in our search for the potential pathways that are influenced by...
DmE25, a traditional Chinese medicinal formula used in cancer treatment.

HSP27, YangZheng XiaoJi and sensitivity to chemotherapeutic agents. The role of HSP27 in the sensitivity to chemotherapy agents is not without controversy. For example, it has been reported recently that overexpression of HSP27 in pancreatic cancer cells would increase the sensitivity of the cancer cells to gemcitabine (37). Although reasons for this are not clear, the type of drugs used in the study may be an explanation, as these drugs are transported and act in cancer cells via different mechanisms.

HSP27 acts as a cyto-protective molecule during androgen independence in prostate cancer patients and in prostate cancer cell models (38). Likewise, levels of HSP27 correlated with resistance to irinotecan in colorectal cancer cells (39). HSP27, together with HSP70 is linked to the sensitivity of colorectal cancer to the naturally-occurring anticancer agent curcumin (40).

Knocking down HSP27 sensitises glioblastoma multiforme tumour cells to the HSP90 inhibitor, 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) and staurosporine by changes in apoptosis (41). The same was seen in glioma cells, in that silencing HSP27 resulted in an increase in apoptosis in response to temozolomide and quercetin (42).

HSP27, YangZheng XiaoJi and cell migration. The finding that HSP27 is co-localised with phalloidin to the focal adhesion

Table III. Effects of 5-FU on the growth of cancer cells in combination with DmE25.

<table>
<thead>
<tr>
<th>5-FU</th>
<th>1.6 µg/ml</th>
<th>40 µg/ml</th>
<th>1000 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>YZXJ</td>
<td>Control</td>
</tr>
<tr>
<td>AGS</td>
<td>13.1±1.6</td>
<td>3.6±1.8</td>
<td>59.6±0.8</td>
</tr>
<tr>
<td>MIAPAC2</td>
<td>NC</td>
<td>57.8±1.5</td>
<td>33.4±5.9</td>
</tr>
<tr>
<td>A549</td>
<td>15.0±3.1</td>
<td>12.7±4.1</td>
<td>59.2±2.0</td>
</tr>
<tr>
<td>SKMES1</td>
<td>10.5±2.3</td>
<td>29.0±1.4</td>
<td>51.9±3.4</td>
</tr>
<tr>
<td>COV504</td>
<td>15.6±1.4</td>
<td>48.2±1.6</td>
<td>40.5±2.4</td>
</tr>
<tr>
<td>SKOV3</td>
<td>10.4±0.8</td>
<td>20.9±2.8</td>
<td>32.7±43.5</td>
</tr>
</tbody>
</table>

NC, negative control.

Figure 7. (A and B) Effects of knocking down HSP27 on the migration of SKMES1 cells. (C) The knockdown also increased the sensitivity of cancer cells to low concentration of drugs, in particular when combined with DmE25. (D) Effects of knocking down HSP27 on the migration of PANC-1 cells which is further decreased in the presence of DmE25.
Table IV. Effects of topotecan on the growth of cancer cells in combination with DME25.

<table>
<thead>
<tr>
<th>Topotecan</th>
<th>3.2 ng/ml</th>
<th>80 ng/ml</th>
<th>2 µg/ml</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>YZXJ</td>
<td>Control</td>
</tr>
<tr>
<td>AGS</td>
<td>7.6±8.6</td>
<td>18.3±3.7</td>
<td>46.0±5.8</td>
</tr>
<tr>
<td>MIAPACA2</td>
<td>0.9±0.9</td>
<td>65.6±4.1</td>
<td>39.5±3.3</td>
</tr>
<tr>
<td>A549</td>
<td>NC</td>
<td>NC</td>
<td>48.9±4.3</td>
</tr>
<tr>
<td>SKOV3</td>
<td>11±0.7</td>
<td>30.2±2.1</td>
<td>38.9±1.1</td>
</tr>
<tr>
<td>COV504</td>
<td>NC</td>
<td>23.1±0.8</td>
<td>24.9±5.1</td>
</tr>
<tr>
<td>SKMES1</td>
<td>7.8±0.6</td>
<td>40.4±2.8</td>
<td>62.3±1.4</td>
</tr>
</tbody>
</table>

Table V. Effects of cisplatin on the growth of wild-type and cisplatin-resistant ovarian cancer cells in combination with DME25.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>A2780</th>
<th>A2780/CP70</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>160 ng/ml</td>
<td>4 µg/ml</td>
</tr>
<tr>
<td>Cisplatin only</td>
<td>12.3±8.2</td>
<td>72.5±2.5</td>
</tr>
<tr>
<td>Cisplatin + YZXJ</td>
<td>72.4±3.6</td>
<td>80.5±3.3</td>
</tr>
</tbody>
</table>

regions of cancer cells is interesting. From the immunofluorescence staining, HSP27 particularly the phosphorylated form of the protein, is frequently co-localised with Phalloidin at the focal adhesion regions of cancer cells, where caspase-9 is also seen. It is of interest to note that inclusion of YZXJ extract DME25 in the study markedly reduce the presence of phospho-HSP27 (S86) at the focal adhesions. Given the key role of focal adhesion in cell-matrix adhesion and cellular migration, it is plausible to suggest that activation of HSP27 (by way of phosphorylation) coordinates with other proteins in the focal adhesion area, such as integrins and the focal adhesion kinase (FAK) to regulate the adhesion and migration of cancer cells. We have recently reported that YZXJ was able to inhibit the phosphorylation of FAK in cancer cells and in endothelial cells (35,43).

Together with the finding that knocking down HSP27 resulted in an increase in cellular migration in cancer cells, it is suggested that HSP27, in particular when combined with YZXJ, is an important regulator of EMT reversal or MET. It has been reported recently that HSP27 is key to IL-6 dependent and IL-6 independent EMT in prostate cancer cells, by acting on IL-6 induced activation of STAT3/Twist and Snail (44,45). It is also involved in EGF induced EMT in prostate cancer cells by influencing the migration of these cells (46,47). Other than the reported effects on cancer cells, HSP27 has also been shown to be a mediator for angiogenesis (48), suggesting that HSP27 is involved in a wider range of processes in cancer development and progression.

Taken together, it is clear that HSP27 is a viable target in cancer treatment and that a combined approach, with either YZXJ or another method, would be a valid consideration when devising a therapy in either targeting HSP27 or in certain chemoresistance cases.

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References


