Impact of *Yangzheng Xiaoji* on the Adhesion and Migration of Human Cancer Cells: the Role of the AKT Signalling Pathway

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Abstract. *Background: Yangzheng Xiaoji is a traditional Chinese medical formulation that has been shown to have anticancer actions in patients with various solid tumours. The mechanisms of the potential anticancer action of Yangzheng Xiaoji are unknown. In the present study, we investigated the direct effects of Yangzheng Xiaoji on a range of human cancer cell lines and investigated the possible mechanism(s) of its action. Materials and Methods: Extract of Yangzheng Xiaoji (DME25) was prepared using dimethyl sulfoxide. The influence of DME25 on in vitro growth, adhesion and migration was examined using in vitro function assays. The effects on signalling protein kinases were assessed using western blotting. Results: DME25 suppressed adhesion and migration of various cancer cell, including those of breast, prostate, lung, osteosarcoma and colorectal cancer. Further investigation showed an involvement of the phosphatidylinositol 3-kinases/protein kinase B (PI3K/AKT) pathway in the inhibitory effect on the adhesion of cancer cells by DME25. Conclusion: Yangzheng Xiaoji exerts its anticancer effects not only via synergistically working together with chemotherapy, but also by directly inhibiting adhesion and migration of cancer cells. The PI3K/AKT pathway is a potential signalling pathway targeted by Yangzheng Xiaoji.*

Traditional Asian medicine has been practised for centuries in China, Korea, Japan, and other countries in Asia. The traditional practice has been used in therapy of a variety of diseases, including cancer. Although limited successe has been achieved from the studies of traditional medicine, such as artemisinin for malaria and arsenic trioxide for leukaemia, to date, most of the traditional remedies are short of scientific explanations for their mode of actions, if any. Traditional Chinese Medicine (TCM) has been used to prevent and treat various disorders with documented records for two thousand years. Acupuncture and herbal remedies have been introduced and practised to some extent in Europe and other areas around the world. Recent studies of TCM in cancer have been focused on molecules extracted and purified from herbs. For example, icariside II purified from the root of *Epimedium koreanum Nakai* induces apoptosis of the human acute myeloid leukaemia (AML) cell line U937 cells *via* signal transducer and activator of transcription 3 (STAT3)-related signalling (1). However, the efficacy and mechanistic investigations into TCM in the prevention and treatment of malignancies, particularly anticancer remedies, remain poor.

Yangzheng Xiaoji is a traditional Chinese medical formula that has been shown to have anticancer actions in patients with various solid tumours. In a recent randomised, double blinded study of patients with primary liver cancer, patients who received conventional chemotherapy, combined with *Yangzheng Xiaoji* (n=304) had a significantly increased rate of disease remission (complete and partial remissions), compared with patients who received chemotherapy alone (n=103) (23.3% *vs*. 14% $p<0.01$) (2). In the study, patients who received the combinational therapy had also improved quality of life, based on the Karnofsky method. The formula has also been reported to be able to improve atypical dysplasia of the stomach (3).

The mechanisms of the potential anticancer action of *Yangzheng Xiaoji* are not clear. It has been shown that patients who received *Yangzheng Xiaoji* and chemotherapy have less bone marrow suppression compared with those who received chemotherapy alone (2). It has been suggested, therefore, that one of the mechanisms

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underlying the clinical observations is that *Yangzheng Xiaoji* may improve the immune function of the body. However, whether the formula has a direct effect on cancer cells is not clear.

In addition to the body's defence, cancer progression is also dependent on the biological characteristics of cancer cells, including the rate of cell proliferation, invasiveness, the ability to degrade matrix and subsequent migration and invasion. Angiogenesis is also vital to the distant spread of cancer cells. The latter cell functions are also closely linked to the metastatic potential of cancer cells. Naturally occurring compounds have been reported to be able to influence a number of these cell functions. For example, taxol, a plant alkaloid, was initially extracted from Western Yew bark and is a widely used chemotherapeutic agent (4). Fumagillin is also a natural product shown to be a strong anti-angiogenic agent (5). Artenisinin, a compound extracted from *Qinhao*, a Chinese medical herb has been widely used in the treatment of malaria and has also been introduced in cancer treatment (6-9).

In the present study, we investigated the direct effect of *Yangzheng Xiaoji* on a range of human cancer cell lines and we report that *Yangzheng Xiaoji* has little effect on the growth of cancer cells, including human breast, colorectal, prostate, lung cancer and osteosarcoma cells. However, the formula had some marked inhibitory effects on the adhesion and migration of the cancer cells. We further report that this action is likely to occur *via* the phosphatidylinositol 3-kinases/protein kinase B (PI3K/AKT) signalling pathways.

Materials and Methods

Materials and cell lines. Human breast cancer cell lines MCF-7 and MDA MB-231, human prostate cancer PC-3 and DU-145, human gastric cancer cell lines HGC27 and AGS, and human colorectal cancer cell lines RKO and HRT18 were obtained from ECACC (European Collection of Animal Cell Culture, Salisbury, UK); human osteosarcoma MG-63 and human lung cancer cell line A549 were purchased from the Americal Type Cell Culture (Teddington, UK). These cells were maintained in Dubecco's Modified Eagle's medium (DMEM) (Sigma-Aldrich, Poole, Dorset, UK) supplemented with penicillin, streptomycin and 10% foetal bovine serum (Sigma-Aldrich). The cells were incubated at 37˚C, 5% CO_2 and 95% relative humidity.

Rho-associated protein kinanse (ROCK) small inhibitor (Y27632) was purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz Biotechnology, Inc., CA, USA). c-Jun N-terminal kinase (JNK) inhibitor II (SP60015), extracellular-signal-regulated kinase (ERK) inhibitor II (FR180204), Janus kinase-3 (JAK-3) inhibitor (Jak-3 Inhibitor 1 {4-(4'-Hydroxyphenyl)amino-6,7-dimethoxyquinazoline; WHI-P131}), and phospholipase C gamma (PLC-γ) (U73122) were purchased from Calbiochem (Merck Chemicals Ltd, Nottingham, UK). Hepatocyte growth factor (MET) kinase inhibitor was obtained from Pfizer Pharmaceutical. Matrigel (reconstituted basement membrane) was purchased from Collaborative Research Products (Bedford, MA, USA). Anti-human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and anti-phopho-AKT (pAKT) antibodies were purchased from Santa Cruz Biotechnology Inc.

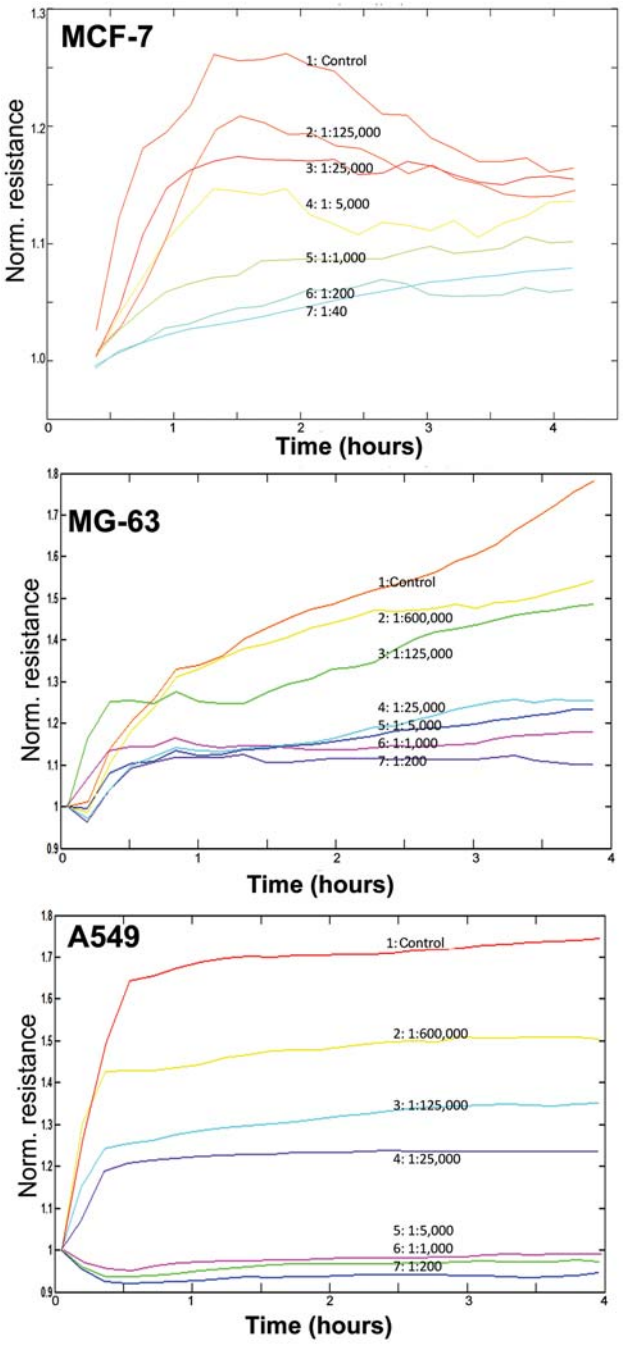

Figure 1. *The effect of DME25 on the matrix adhesion of MCF-7 (top), MG-63 (middle) and A549 cells (bottom). Adhesions were inhibited at concentrations higher than 1:125,000.*

Preparation of extract from Yangzheng Xiaoji for experimental use. Medicinal preparation of *Yangzheng Xiaoji* (Yiling Pharmaceuticals,

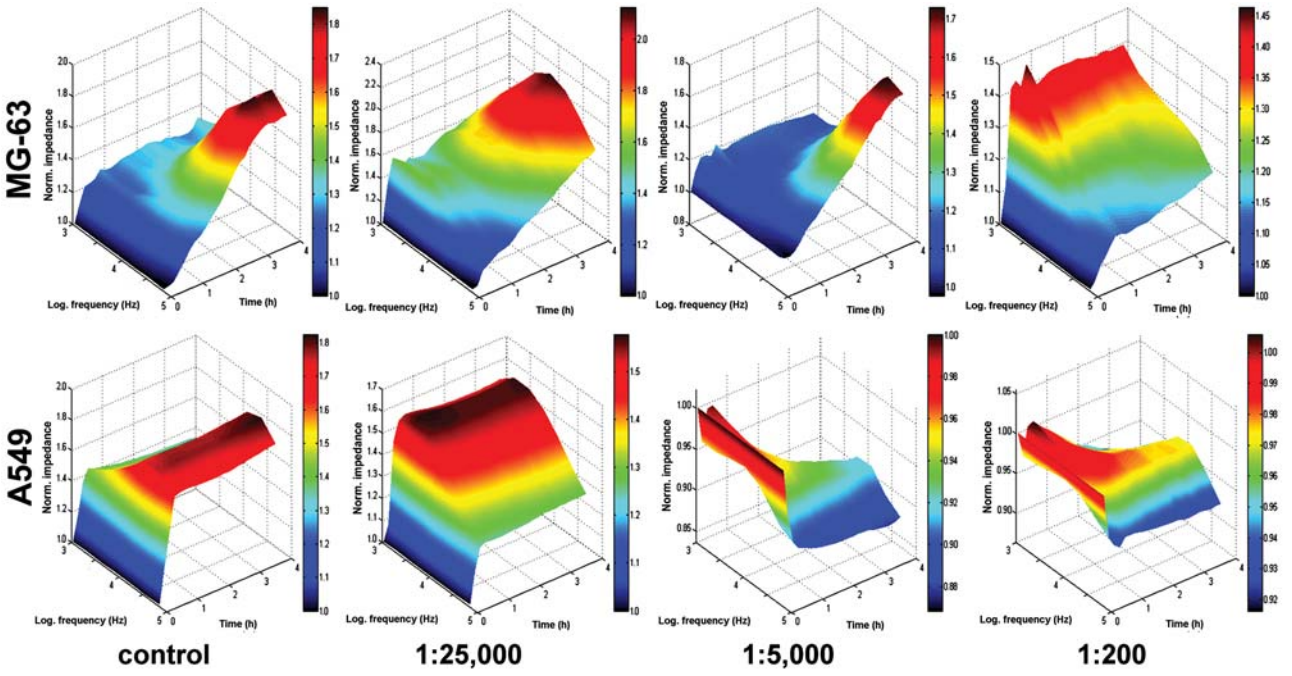

Figure 2. *DME25 inhibited cell adhesion, as detected over a broad spectrum from frequencies. 3D models were used to demonstrate the effect of the extract on cell adhesion, in which the X-axis displays frequencies, the Y-axis resistance and the Z-axis time.*

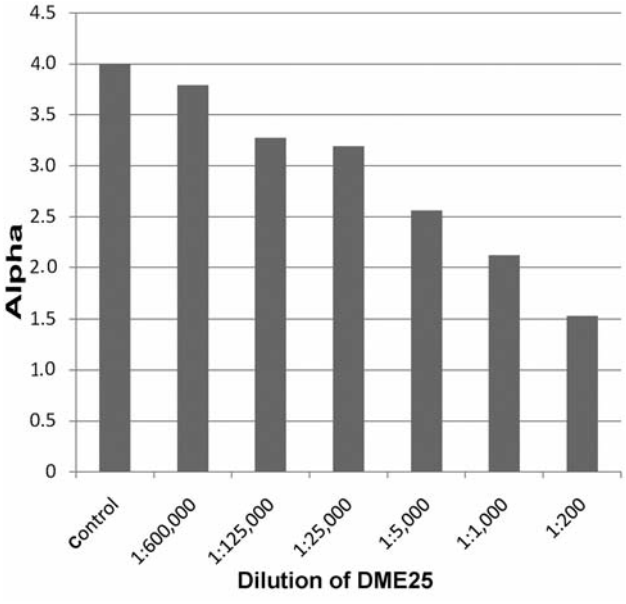

Figure 3. *Rb modelled data from A549 cells.*

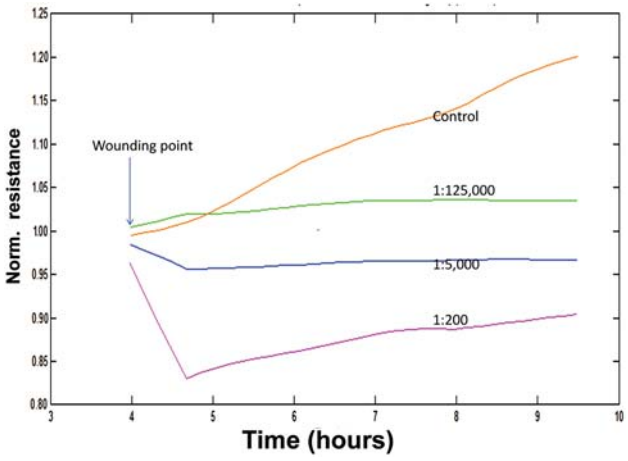

Figure 4. *DME25 inhibited cell migration in A549 cells.*

Shijiazhuang, Hebei, P.R. China) was subject to extraction using DMSO, balanced salt solution and ethanol respectively, on a rotating wheel for 12 hours at 4˚C, as we recently reported (10). Insolubles were removed after centrifugation at 15,000 ×*g*. The DMSO preparation was found to be more effective and with better yield compared with the other two methods. DMSO extract was hence used in the subsequent experiments. The extract was standardised by quantifying the optical density of the preparation using a spectrophotometer at 405 nm wavelength. A master preparation of 1:1000 diluted extract which gave 0.25 OD was stocked as the master stock and was named as DME25 for the experiments.

In vitro cell growth assay. Cell growth was assessed using an *in vitro* growth assay (11, 12). Cells were seeded into 96-well plates

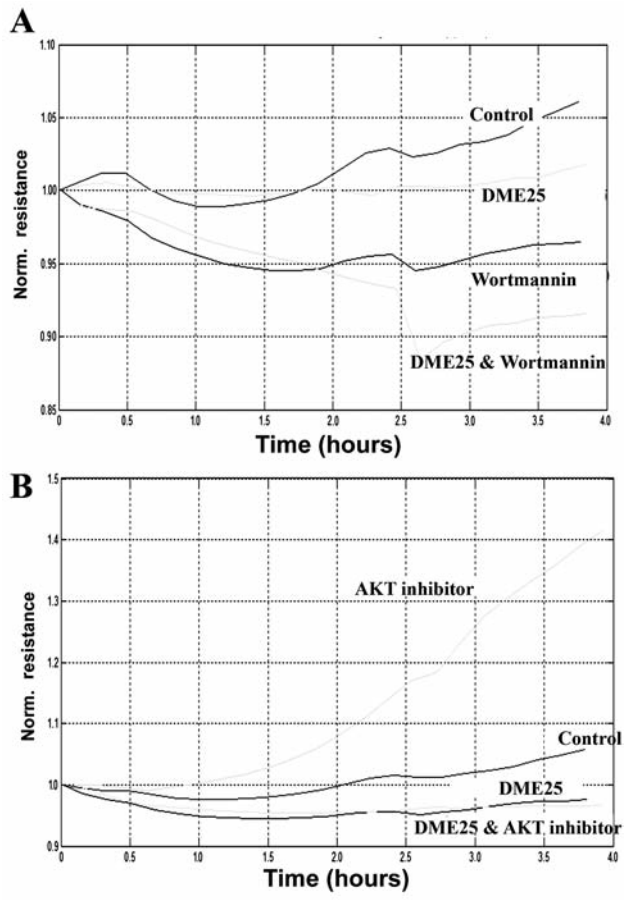

Figure 5. *Effect of the phosphatidylinositol 3-kinases/protein kinase B (PI3K/AKT) pathway on DME25 induced cell adhesion in DU-145 cells. A: Influence on cell adhesion by addition of PI3K inhibitor wortmannin. B: AKT inhibitor and its implication on the effect on cell adhesion by DME25.*

at a density of 3,000 cells/well. The cells were treated with DME25 of different dilutions, 6 wells for each group including a control group. Triplicate plates were set-up for overnight, 3-day and 5-day incubation periods. Following the incubation, the plates were removed from the incubator, fixed in 4% formaldehyde (v/v), and stained with 0.5% (w/v) crystal violet. The crystal violet stain was subsequently extracted using 10% acetic acid (v/v), allowing for the detection of cell density through spectrophotmeric analysis of the absorbance of the resultant solution, using a Bio-Tek ELx800 multi-plate reader (Bio-Tek Instruments Inc, VT, USA).

Electric cell-substrate impedance sensing (ECIS)-based cellular adhesion and migration assays. The ECIS-Zθ instrument (Applied Biophysics Inc, NJ, USA) was used for cell adhesion and motility (wounding assay) assays in this study (13, 14). Cell modelling was carried out using the ECIS RbA modelling software, supplied by the manufacturer. The 96W1E ECIS arrays were used in the present study. ECIS measures the interaction between cells and the substrate to which they attached *via* gold-film electrodes placed on the surface of culture dishes. Following a pre-treatment of the array surface with a cysteine solution (10 mM), the arrays were incubated with complete medium for 1 hour. The same number of the cells (30,000 cell/well) was added to each well. In the cell adhesion assay, the adhesion was tracked immediately after adding the cells into the arrays. For cell migration assay, the arrays with the cells were allowed to reach confluence after 3 hours. The monolayer of the cells was electrically wounded at 2,000 μA for 20 sec. Impedance and resistance of the cell layer were immediately recorded for a period of up to 20 h. For signalling transduction inhibitor assays, the respective inhibitors were included in the assay wells. Adhesion and migration were modelled using the ECIS RbA cell modelling software (15).

Western blot analysis. Cells were grown to confluence in a 25-cm^3 tissue culture flask, detached and lysed in HCMF buffer containing 1% Triton X-100, 2 mM $CaCl_2$, 100 μg/ml phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml aprotinin and, 10 mM sodium orthovanadate on a rotating wheel for 1 h before being spun at 13,000 ×*g* to remove insolubles. The protein levels in the samples were subsequently quantified using the Bio-Rad DC Protein assay kit (Bio-Rad Laboratories, CA, USA). Equal amounts of proteins were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Once sufficient separation had occurred the proteins were blotted onto a Hybond-C Extra nitrocellulose membrane (Amersham Biosciences UK Ltd, Bucks, UK), blocked in 10% milk and probed for the expression of specific proteins. Phosphorylated AKT (threonine) expression was detected using the anti-pAKT antibody (Santa Cruz Biotechnology, Inc.). In addition to this, GAPDH expression was also assessed using an antibody specific to this molecule (Santa Cruz Biotechnology, Inc.), in order to assess total protein levels and uniformity of the test samples. Following specific binding of the primary antibody, the membranes were probed with peroxidase conjugated anti-mouse (pAKT and GAPDH) antibody (Sigma, Dorset, UK). Protein signal was then visualised through the Supersignal West Dura Extended Duration substrate chemiluminescent system (Perbio Science UK Ltd, Cramlington, UK) and detected using a UVIProChem camera system (UVItec Ltd, Cambridge, UK).

Results

Effects of Yangzheng Xiaoji on the adhesion of human cancer cells. We tested the effect of DME25 on cell matrix adhesion on cell lines from a number of tumour types, including breast, prostate, lung, colorectal, gastric and osteosarcoma cells, using a high-throughput ECIS method. This was done over a wide range of concentrations (1:40 to 1:600,000). DME25 had an inhibitory effect on cell adhesion, mostly from concentrations higher than 1:125,000. The most sensitive cells tested were MCF-7, MG-63 and A539, which are shown in Figure 1. The inhibitory effects can be demonstrated over a wide spectrum, tested using the current model (1000 to 32,000 Hz). Selected modelling graphs are shown in Figure 2.

Figure 3 shows an example (A549 cell) of quantitative analysis of the adhesion using the Rb method. The parameter

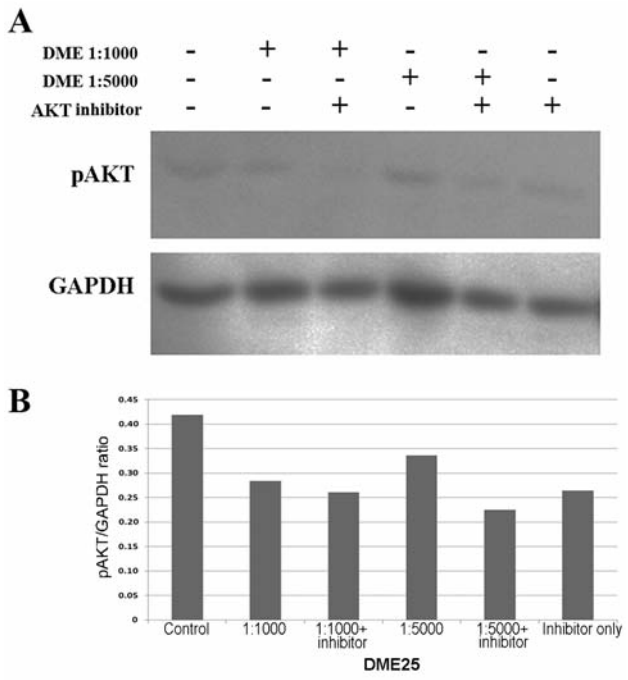

Figure 6. *Effect on phosphorylation of protein kinase B (AKT) (Ser 473) by DME25 in A549 cells. A: Western blot shows an effect on phosphorylated AKT by DME25. B: Quantification of the bands was performed using ImageJ. Bar graph shows pAKT/GAPDH ratio.*

Alpha is used to indicate the average distance between the basal membrane of the cells and the electrode (the substrate). Therefore it clearly demonstrates a concentration-dependent inhibition of DME25 on the cell-substrate adhesion of the tested cancer cells.

Yangzheng Xiaoji inhibited cell migration. Following the tests of the effect on cancer cell adhesion, we carried out electrical wounding on the confluent monolayer of cancer cells. The resultant wounds were healed by the surrounding cancer cells which were being monitored by the ECIS instrument. DME25 exhibited an inhibitory effect on the migration of cancer cells in a concentration-dependent manner, affecting lung cancer and colorectal cancer cells. Figure 4 shows the effect of DME25 on the migration of A549 cells. The inhibitory effect was seen at a concentration as low as 1:125,000, similar to that seen on cell adhesion.

Yangzheng Xiaoji had little influence on the growth of human cancer cells. We further analysed the effect of DME25 on cell growth *in vitro*. Cell growth results following 72 h treatment with DME25 (1:1000 and 1:25,000) are shown in Table I. Over the broad concentration range tested, there was no significant effect on the growth of cancer cells observed due to exposure to DME25.

Table I. *Effect of DME25 on the in vitro growth of cancer cells.*

Cell type	Absorbance (490 nm)		
	Control (0)	1:1,000	1:25,000
RKO, colorectal cancer	0.82±0.31	0.94±0.15	1.01±0.10
PC-3, prostate cancer	0.39±0.11	0.37±0.08	0.35±0.05
MCF-7, breast cancer	1.39±0.34	1.52±0.41	1.32±0.54
HGC27, gastric cancer	0.72±0.10	0.95±0.31	0.97±0.10
A549, osteosarcoma	0.24±0.08	0.21±0.07	0.24±0.03

PI3K/AKT pathway in Yangzheng Xiaoji-mediated cell adhesion. To explore pathways involved in the effect of DME25 on cell adhesion, we tested the effect of inhibitors targeting PI3K and AKT, respectively. Addition of wortmannin (PI3K inhibitor) alone or in a combination with DME25, reduced adhesion of prostate cancer DU-145 cells to an even lower level compared to both control and cells exposed to DME25 alone (Figure 5A). The combination of wortmannin and DME25 had a further inhibition on cell adhesion in comparison with treatments of either of the two materials. The inhibitor of AKT, a downstream pathway in the PI3K pathway together with DME25 did enhance the inhibitory effect on cell adhesion. Interestingly, the inhibitor itself affected the cell adhesion in a different way which appeared to be increased.

Figure 6 shows the influence of DME25 on phosphorylation of AKT (Ser 473) in A549, a lung cancer cell line. DME25 at a higher concentration (1:1,000 dilution) reduced the level of pAKT. Addition of AKT inhibitor and DME25, synergistically reduced the pAKT, particularly with lower concentration of DME25 (1:5,000 dilution).

Discussion

Molecules or compounds extracted from natural products (plants or herbs) have been utilised to treat cancer. Over the last two decades, some studies have been carried out to examine the mechanisms of these natural molecules or compounds to affect cancer cells. However, in the traditional Asian medicine, particularly TCM, most of the commonly used remedies are composed of multiple herbs. One of the modified traditional remedies from TCM to treat cancer is *Yangzheng Xiaoji* capsule, which has been proven as a remedy that can synergistically work together with chemotherapy to improve the treatment of liver cancer (2, 3). Effects on the host immune system have been indicated as part of the therapeutic effect of *Yangzheng Xiaoji*. The direct effect and the relevant mechanisms of Yangzheng Xiaoji action remain unknown. The present study is therefore to our knowledge, the very first to show a direct effect of DME25 on various cancer cell lines.

We firstly examined the effect of *Yangzhang Xiaoji* on *in vitro* growth of various cancer cells derived from different solid tumours or metastases, including breast, prostate, lung, gastric and colorectal cancer cells, and also osteosarcoma cells. The experimental results did not show any obvious impact on the *in vitro* growth of these tested malignant cells. This suggests that the anticancer effect of *Yangzheng Xiaoji* most probably does not rely on direct inhibition of the growth of cancer cells.

In addition to unlimited growth, traits of cancerous cells include adhesion, migration and invasion, which are essential for them to leave the primary tumour, disseminate throughout the body, either locally or spread to a distant organ *via* lymphatic, blood vessels or body cavities, and eventually develop into metastases.

Further experiments were therefore performed to determine the impact of *Yangzheng Xiaoji* on such capacities of cancer cells. DME25 exhibits a concentration-dependent effect on adhesion of the tested cancer cells. For example, such an effect was seen in A549, a lung cancer cell line, on exposure to DME25 from a concentration as low as 1:600,000. Wounding assays also demonstrated that *Yangzheng Xiaoji* had an inhibitory effect on the migration of the cancer cells.

Further experiments demonstrated an involvement of the PI3K pathway in the effect of *Yangzheng Xiaoji* on the adhesion of cancer cells by. Apart from the established role in cell proliferation and survival, the PI3K pathway has also been reported to play a role in the regulation of cell adhesion and migration (16-19). Wortmannin, a potent inhibitor of PI3K activation, can suppress the activation of PI3K on AKT and mammalian target of rapamycin (mTOR) (20, 21). Wortmannin can enhance the inhibition of adhesion by DME25. The addition of an AKT inhibitor alone exerted a different effect. It suggests that the involvement of the PI3K pathway in the effect of DME25 does not only rely on a single pathway, *e.g.* AKT pathway.

In conclusion, *Yangzheng Xiaoji* exerts its anticancer effects *via* directly inhibiting the adhesion and migration of cancer cells. The PI3K/AKT pathway is involved in the effects of *Yangzheng Xiaoji*. Future investigations will shed further light on the mechanisms of the anticancer effect of *Yangzheng Xiaoji*, and may help to improve its therapeutic application.

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