Combined use of *Andrographis paniculata* and chemotherapeutics for metastatic oesophageal cancer: a pre-clinical study

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KEY MESSAGES

1. The combined use of water extract of *Andrographis paniculata* (APW) and chemotherapeutics (cisplatin plus 5-fluorouracil) reduced the metastasis of oesophageal tumour to lung in mouse models.
2. The combined use of APW and chemotherapeutics significantly suppressed the oesophageal xenograft growth by enhancing apoptosis.
3. Combined use of APW and chemotherapeutics had additional immunomodulatory benefit of APW.
4. The absorbed components of APW possessed anti-migratory activities in oesophageal cancer cells.

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Introduction

Oesophageal cancer (OC) is the fourth and sixth leading cause of cancer-related death in China and United States, respectively. Surgery is the main treatment option for OC but has high recurrence rate owing to metastasis. Surgery followed by adjuvant chemotherapy and concurrent radiotherapy is a more effective treatment. The standard chemotherapeutics are cisplatin and 5-fluorouracil (5-FU).[1] Nevertheless, chemotherapy may cause a number of adverse effects that hamper efficacious treatment.

Many cancer patients use herbal prescriptions and supplements to combat cancer, strengthen the immune system, and counter some side-effects of the conventional treatments. A systematic review on Chinese herbal medicine for OC has shown improvement in immune system, extension of survival, reduction of adverse reactions to chemotherapy and radiotherapy, and the holistic function of the patients.[2] More scientific evidence of using Chinese herbal medicine for metastatic OC should be further explored.

The water extract of *Andrographis paniculata* (AP) has been shown to have anti-tumour effects in human OC cells. This study aimed to evaluate the anti-tumour and anti-metastatic activities of AP water extract (APW) combined with OC chemotherapeutics (cisplatin and 5-fluorouracil) in an OC metastatic mouse model. The immunomodulatory effect of APW was investigated in immune-competent mice. The gastrointestinal absorption characteristics of APW were determined in a human intestinal Caco-2 cell transport model, which is a pre-clinical integral component of the Biopharmaceutics Classification System and can be used to investigate the gastrointestinal absorption, permeability, and drug-drug interactions.[4] The anti-metastasis effects of the absorbed AP components (AAPC) through the Caco-2 transport model were verified in human oesophageal cancer cells in vitro.

Materials and Methods

A single lot of dried whole plant of AP (about 10 kg) was purchased from a renowned supplier in Hong Kong. Morphological and chemical authentications were accomplished in accordance with the Chinese Pharmacopoeia 2010. Authenticated voucher specimen (number: 3435) was deposited in the museum of the Institute of Chinese Medicine, CUHK. Dried whole plant of AP was extracted under reflux using water for 1 h and the extraction was repeated once. Following filtration, the crude water extract was centrifuged to remove undissolved particles. The extract was freeze-dried into powder.

The human OC cells EC-109 were obtained from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Beijing, China) and human intestinal Caco-2 cells were obtained from the American Type Culture Collection (MD, USA). Cell culture media and supplements were purchased from Life Technologies (NY, USA). BALB/c nude mice and C57BL/6 mice were provided by Laboratory Animal Services Centre, The Chinese University of

Hong Kong, and were housed under pathogen-free conditions. The experiments were approved by the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong (Ref. No. 12-078-MIS).

Nude mice (6-8 weeks of age) were inoculated intraperitoneally with 5 $\times 10^6$ EC-109 cells in 200 μL PBS on day 0. On day 1, animals were randomised into four groups: (1) control, (2) APW (1600 mg/kg), (3) combination of cisplatin (1.5 mg/kg) and 5-FU (42.5 mg/kg), and (4) combination of APW (1600 mg/kg), cisplatin (1.5 mg/kg), and 5-FU (42.5 mg/kg). APW was orally administered daily for 21 days, and cisplatin and 5-FU were injected intraperitoneally on days 13 and 19. On day 22, the mice were anesthetised and whole blood was obtained by cardiac puncture. The animals were sacrificed by cervical dislocation. Lungs of the mice were dissected out after cervical dislocation and fixed in 10% buffered formalin. The samples were stained with haematoxylin and eosin. Stained sections were examined and photographed under a light microscope (Olympus IX71, Japan). Evaluation of lung metastasis was carried out according to a previous study.[3]

Nude mice (6-8 weeks of age) were inoculated subcutaneously into the flank with EC-109 (1 $\times 10^6$ cells per 100 μL PBS) on day 1. The mice were randomised into the four groups when the tumour reached the volume of 70 mm^3. Treatment period was 21 days, with the same treatment protocol mentioned above. Body weight was monitored and the size of tumour was measured with a caliper every 3-4 days and were calculated with the formula: $(\text{length} \times \text{width})^2/2$. At the end of treatment (day 22), tumours were excised from mice after sacrifice. Sections were subjected to TUNEL staining to determine the number of apoptotic cells. Blinded assessments were performed in four randomly chosen sections from each mouse using Image J software (NIH).

Immune responses of mice towards APW and/or chemotherapeutics were evaluated in a carcinogen-induced oesophageal dysplasia mouse model (protocol modified from Tang et al.[5]). In brief, C57BL/6 mice (4 weeks of age) were fed ad libitum with a zinc-deficiency diet (Envigo, CA, USA) for 3 weeks and then provided with drinking water containing 60 μg/mL 4-nitroquinoline 1-oxide (NQO, Sigma, MO, USA) for 11 weeks. Treatments started at week 8 and the treatment protocol was the same as that mentioned above. At the end of treatment, the animals were sacrificed and spleens were excised. The isolated spleen leucocytes were subjected to T-cells subsets (eg $CD3e^+$, $CD4^+$, $CD8^+$) measurement using flow cytometry.

Caco-2 cells were seeded at 3 $\times 10^5$ cells/well in six-well plates with Transwell inserts (0.4 μm pore size; Corning, USA) and cultured for 21 days prior to transport experiments. Transport experiments were carried out as described previously.[4] The APW at 400, 800, 1600, 3200, or 6400 μg/mL were added on the apical side of the monolayer and incubated at 37°C for 2 h. The buffer in the basolateral compartment that contains the AAPC was collected and blow dried by termovap nitrogen sample concentrator and redissolved in culture medium for cell assays.

The viability of EC-109 cells was examined by trypan blue exclusion assay. Briefly, EC-109 cells (2.5 $\times 10^5$/well) were seeded in 24-well plates and allowed adhesion overnight. The medium was changed to 10% v/v FBS medium with AAPC (APW at 800, 1600, and 3200 μg/mL) for 48 h. Subsequently, the adherent cells and floating cells were collected. The cell suspension was mixed with trypan blue dye. The number of viable cells was counted under a light microscope (Olympus IX-71, Japan). The motility of EC-109 was assessed by the scratch wound assay as described in previous study.[3] In brief, EC-109 cell layers scraped with wounds were incubated with medium containing AAPC (APW at 800, 1600, and 3200 μg/mL) for 24 h and each well was photographed at 40× magnification under a light microscope. The wound area was blindly assessed using Image J software (NIH).

Data were expressed as mean ± standard deviation for in vitro studies, and as mean ± standard error of the mean for in vivo studies. One-way analysis of variance followed by post-hoc Dunnett test were used to compare the treatment groups and the control group. One-way analysis of variance followed by post-hoc Tukey multiple comparison test were used to determine significant differences among all groups. Statistical analyses were conducted using GraphPad Prism 5.0 (GraphPad Software Inc, San Diego, CA, USA). In all comparisons, $P<0.05$ was considered statistically significant.

Results

Lung metastasis was observed after intraperitoneal inoculation of EC-109 cells for 22 days. Tumours were found in lung sections; treatments with APW, cisplatin plus 5-FU, or both could inhibit the lung metastasis in intraperitoneal xenograft-bearing nude mice (Fig 1).

Combined use of APW with cisplatin plus 5-FU showed inhibitory effects on EC-109 tumour growth (Fig 2). There was no significant change in body weight in all treatment groups. The number of the apoptotic cells was significantly higher in combined treatment groups than that in control group.

The number of cytotoxic T-lymphocytes ($CD3e^+$, $CD8a^+$) and T-helper lymphocytes ($CD3e^+$, $CD4^+$) from spleen increased after combined treatment of APW and cisplatin plus 5-FU. The number of cytotoxic T-lymphocytes in combined treatment group was significantly greater than in chemotherapeutic alone group.

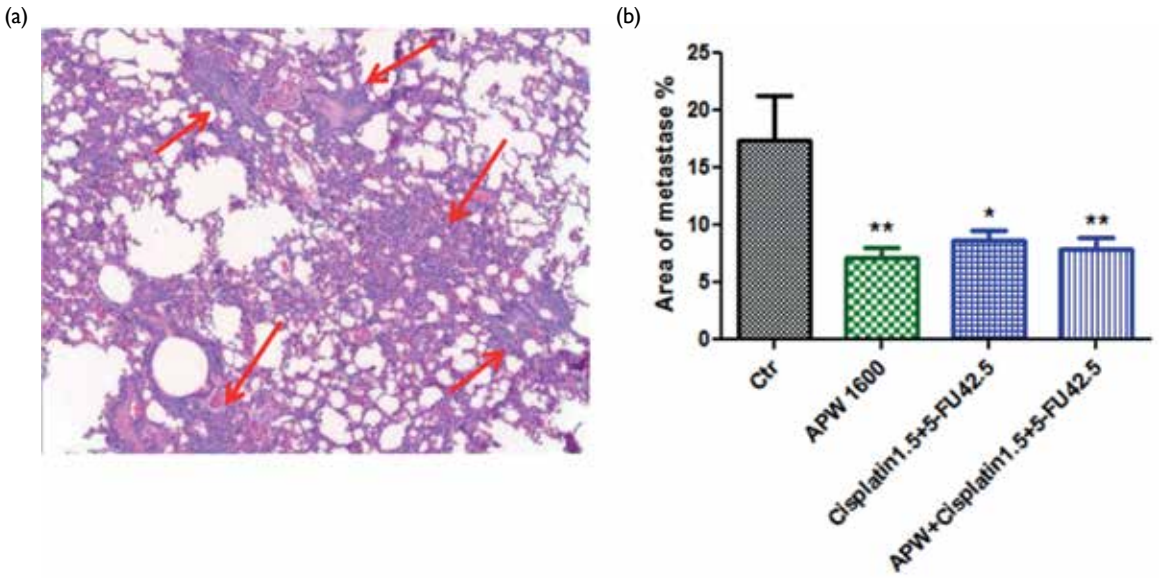

FIG 1. Effects of treatments in EC-109 intraperitoneal xenograft-bearing nude mice. (a) Photos of lung sections showing metastasised tumours (arrows). (b) Tumour burden (area) in lungs as a percentage of whole organ area.

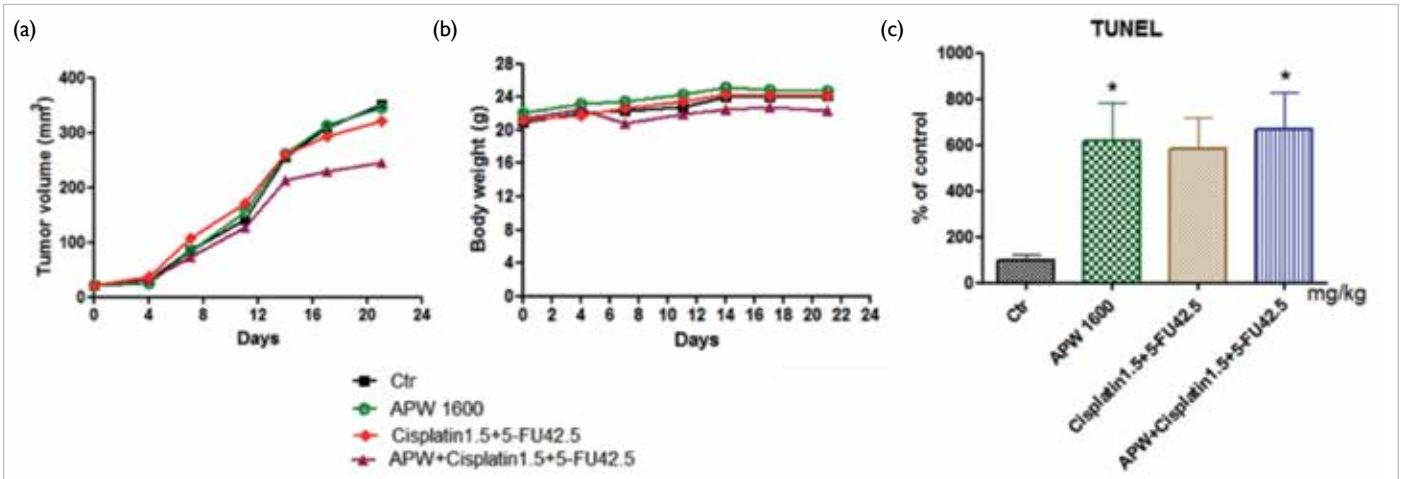

FIG 2. Effects of treatments on (a) tumour size, (b) body weights, and (c) the number of TUNEL-positive stained cells expressed as folds of control in subcutaneous xenograft-bearing nude mice.

AAPC from 800, 1600, and 3200 μg/mL APW did not affect the viability of EC-109 cells after 48 h incubation (Fig 3). The open wound area in wells treated with AAPC from 800, 1600, and 3200 μg/mL APW were larger than those of control wells after incubation for 24 h, suggesting the cell motility was suppressed by AAPC.

Discussion

The present studies involved three mouse models. The intraperitoneal xenograft-bearing nude mice model demonstrated the anti-metastatic activities of APW and/or chemotherapeutics. The effects may be responsible for the prolongation of survival attained after the combined treatment (data not shown). The enhanced anti-tumour effects in combined treatment were observed. The combined treatment significantly increased the number of apoptotic cancer cells in tumour. A reduction of number of proliferative cancer cells was observed in tumour sections from combined treated mice (data not shown), suggesting the proliferation of cancer cells was also inhibited by combined treatment.

To investigate the immunomodulatory effect of APW, the NQO-induced oesophageal dysplasia mouse model showed significant alterations of T-cell populations after treatments. The combined

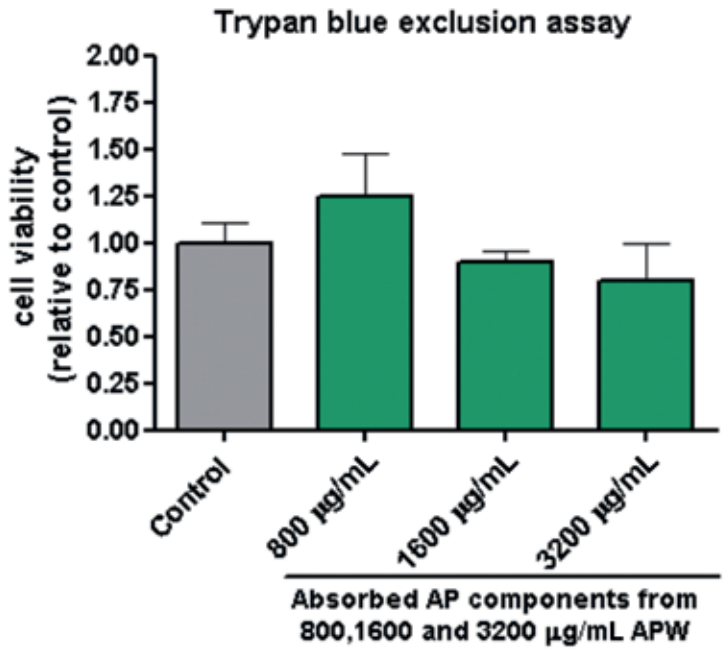

FIG 3. Effects of absorbed *Andrographis paniculata* components on viability of EC-109 cells.

treatments significantly increased T cytotoxic lymphocytes. The cytokine productions from spleen lymphocytes of mice were affected by the APW and/or chemotherapeutics treatments.

The in vitro activities of APW on metastasis were investigated. The bioavailability of andrographolide, which was expected to be the active component of AP, was reported to be poor. Nonetheless, the absorption of the water extract of AP, which is the traditional way to use Chinese herbal medicine, has not been reported. The Caco-2 transport model was used to mimic the gastrointestinal absorption and permeability and to investigate the absorption of APW. The absorbed AP components were found to suppress the motility of OC cells without cytotoxicity. Cancer cell motility and invasion are essential processes in cancer metastasis. These findings suggested that the absorbed components of APW are capable to inhibit the metastasis of cancer cells in vitro.

Conclusion

Combined use of APW and chemotherapeutics exerted anti-tumour and anti-metastatic effects in metastatic OC mouse models, with additional immunomodulatory benefit of APW observed. The in vitro anti-migratory activity of absorbed components of APW could partly explain the efficacies in mouse models. Taking together, the combined treatment (APW plus cisplatin and 5-FU) has more beneficial effects against metastatic OC than chemotherapeutics alone.

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Results from this study have been published in:

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