Antimetastatic activity isolated from *Colocasia esculenta* (taro)

Namita Kundu[a,b], Patricia Campbell[c], Brian Hampton[d], Chen-Yong Lin[a], Xinrong Ma[a], Nicholas Ambulos[c], X. Frank Zhao[b], Olga Goloubeva[a], Dawn Holt[a] and Amy M. Fulton[a,b,e]

Breast cancer mortality is primarily due to the occurrence of metastatic disease. We have identified a novel potential therapeutic agent derived from an edible root of the plant *Colocasia esculenta*, commonly known as taro, which has demonstrable activity in a preclinical model of metastatic breast cancer and that should have minimal toxicity. We have shown for the first time that a water-soluble extract of taro (TE) potently inhibits lung-colonizing ability and spontaneous metastasis from mammary gland-implanted tumors, in a murine model of highly metastatic estrogen receptor, progesterone receptor and Her-2/neu-negative breast cancer. TE modestly inhibits the proliferation of some, but not all, breast and prostate cancer cell lines. Morphological changes including cell rounding were observed. Tumor cell migration was completely blocked by TE. TE treatment also inhibited prostaglandin E_2 (PGE_2) synthesis and downregulated cyclooxygenase 1 and 2 mRNA expression. We purified the active compound(s) to near homogeneity with antimetastatic activity comparable with stock TE. The active compound with a native size of approximately 25 kDa contains two fragments of nearly equal size. The N-terminal amino acid sequencing of both fragments reveals that the active compound is highly related to three taro proteins: 12-kDa storage protein, tarin and taro lectin. All are similar in terms of amino acid sequence, posttranslational processing and all contain a carbohydrate-binding domain. This is the first report describing compound(s) derived from taro that potently and specifically inhibits tumor metastasis. *Anti-Cancer Drugs* 23:200–211 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2012, **23**:200–211

Keywords: antimetastatic activity, breast cancer, cancer therapy, taro, tumor

[a]Marlene and Stewart Greenebaum Cancer Center, [b]Department of Pathology, [c]SOM Genomics Core Facility, [d]SOM Center for Vascular and Inflammatory Diseases, University of Maryland School of Medicine and [e]Baltimore Veteran's Administration Medical Center, Baltimore, Maryland, USA

Correspondence to Amy M. Fulton, PhD, 655 W. Baltimore Street, 10th floor, Rm. 43, Baltimore, MD 21201, USA
Tel: +410 706 6479; fax: +410 706 3260;
e-mail: afulton@umaryland.edu

Received 15 June 2011 **Revised form accepted** 2 August 2011

Introduction

Breast cancer is the second leading cause of cancer death in women in the United States. Breast cancer mortality is primarily due to the occurrence of metastatic disease. Several agents derived from foods have demonstrable chemopreventive and chemotherapeutic activities by multiple mechanisms including enhanced detoxification of carcinogenic intermediates, inducing apoptosis, perturbing cell cycle progression, and inhibiting angiogenesis and metastasis [1–7]. Research into food-derived bioactive components for cancer prevention and cancer therapy is growing because of the relatively low or no-detectable toxicity and better bioavailability.

We have identified and purified potentially novel therapeutic compound(s) derived from an edible root of the plant *Colocasia esculenta*, commonly known as taro. Using two highly metastatic, estrogen receptor, progesterone receptor and Her-2/neu-negative murine mammary tumor cell lines (66.1, 410.4) transplanted to immune competent syngeneic mice, we have now shown that a water-soluble extract of the raw taro corm (TE) can significantly inhibit the lung-colonizing ability and the spontaneous metastasis of these cells. Protective effects are also observed in a therapy model, in which treatment is initiated after tumors are established. It is well established that cyclooxygenase-2 (COX-2) and its product prostaglandin E_2 (PGE_2) are associated with aggressive breast cancer [8,9]. In our previous study, we have shown that pharmacological inhibition of both COX-1 and COX-2 reduces tumor cell proliferation, PGE_2 synthesis, tumor growth and more importantly metastasis in a murine model of breast cancer [10–12]. We now report that TE inhibits PGE_2 synthesis and downregulates COX (1 and 2) mRNA expression. Migration *in vitro* is also inhibited by TE. The addition of TE to some human and murine cancer cell lines profoundly affects cellular morphology and cell proliferation in a dose-dependent manner. Others have shown that cooked mashed corm of the taro plant, known as poi, has antiproliferative activity against the rat YYT colon cancer cell line *in vitro* [13]. We have tentatively identified the active compound(s) as three closely related proteins. Besides our own preliminary data, little is known regarding the potential anticancer or antimetastatic activity of taro *in vivo*.

0959-4973 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e32834b85e8

Methods

Ethics statement

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine (Institutional Animal Care and Use Committee protocol 0708005) and were carried out in strict accordance with the recommendations in the guide for the Care and Use of Laboratory Animals of the National Institutes of Health (USA).

Mice

Syngeneic Balb/cByJ female mice were purchased from Jackson Laboratories (Bar Harbor, Maine, USA). All mice were housed in microisolator cages, fed conventional, autoclaved chow and provided drinking water *ad libitum*.

Taro extract preparation

Commercially obtained taro corm was peeled, combined with phosphate-buffered saline (PBS) in a weight: volume ratio of 1 : 3, blended at low speed, followed by high speed to liquefy. After centrifugation at 1200 rpm for 15 min at 4°C, the supernatant was subjected to high-speed centrifugation (15 000 rpm, 20 min at 4°C) and filter sterilized. The protein concentration of the stock TE was determined using Coomassie Plus Protein Assay Reagent (Pierce Biotechnology, Rockford, Illinois, USA) and ranged from 1.69 to 3.43 mg/ml. For the following experiments, stock TE of 2 μg/μl protein was used, unless otherwise indicated.

Isolation of active components

Stock TE was centrifuged through Amicon Ultra 10 K (10 000) nominal molecular weight limit devices (Millipore Corporation, Billerica, Massachusetts, USA), at 4000 g for 45 min at 25°C. After filter sterilization, the high-molecular weight fraction and the low-molecular weight fraction were used without further treatment.

Column chromatography and electrophoresis

Preparative size exclusion chromatography (SEC) was performed on a Biosuite 250, 13 μ, 21.5 × 300 mm column (Waters Corp., Milford, Massachusetts, USA) using Dulbecco's PBS with calcium and magnesium, at a flow rate of 2 ml/min. Fractions (0.5 min) were collected and pooled based on UV absorbance at 220 nm. Preparative anion-exchange chromatography was performed on an HQ/20, 10 × 100 mm column (Applied Biosystems Inc., Foster City, California, USA), using a 30-min gradient of 0–30% B at a flow rate of 5 ml/min: buffer A = 50 mmol/l of Tris, pH 8.0; buffer B = 50 mmol/l of Tris pH 8 and 1.0 mol/l of NaCl. Fractions (0.5 min) were collected and pooled based on UV absorbance at 220 nm. The pooled samples were concentrated using Centricon Plus 70 10K nominal molecular weight limit devices (Millipore Corporation). Buffer exchange was carried out using Zeba Desalt Spin Columns, Pierce Protein Research Product (Thermo Scientific, Rockford, Illinois, USA). Analytical reversed-phase liquid chromatography (RPLC) was performed using a Jupiter C5 300 Å column (Phenomenex, Torrance, California, USA), using a 40-min gradient of 1–100% B at 1 ml/min: buffer A = 0.1% trifluoroacetic acid (TFA) in water; buffer B = 0.1% TFA in water:acetonitrile (20 : 80) with UV detection at 215 nm. All chromatography was carried out on Beckman Coulter HPLC systems with System Gold V8 or 32 Karat software packages (Beckman Coulter Inc., Fullerton, California, USA). Isolated proteins were further purified using reversed-phase high-performance liquid chromatography (rpHPLC) on a Waters 2695 HPLC system (Waters Corporation, Milford, Massachusetts, USA). Absorbance was monitored using an Applied Biosystems 785 UV detector at 214 nm. Proteins were separated on a Waters Symmetry 300 3 μ C4 1 mm × 150 mm column with a gradient of 0.1% TFA in water (solvent A) and 0.09% TFA in acetonitrile (solvent B). Automated Edman degradation was performed on an Applied Biosystems 494 HT. Proteins were identified by a similarity search of the nr database using the BLASTP 2.2.24+ algorithm via the National Center of Biotechnology Information web interface [14]. Sub fraction 1.1 was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions.

Tumor cell lines

Two murine mammary tumor cell lines (66.1 and 410.4) [15] were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products Inc., Calabasas, California, USA), 2 mmol/l of glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml) and 0.1 mmol/l nonessential amino acids in a 10 and 5% CO_2 humidified atmosphere, respectively. The nontumorigenic murine mammary epithelial cell line EpH4 derived from a Balb/c mouse was maintained in the growth medium described for murine tumor cell lines but without nonessential amino acids and in a 5% CO_2 atmosphere. Human breast cancer cell lines MCF-7 [16], MDA-MB-231[17] and T47D [16] were cultured in the growth medium described for murine tumor cell lines but in a 5% CO_2 atmosphere and MDA-MB-435 [17] cells were grown in minimal essential medium with Earle's salt, L-glutamine (2 mmol/l), sodium pyruvate (1 mmol/l), nonessential amino acids (0.1 mmol/l), vitamins (2X) and 5% fetal calf serum. MCF10A [18], an immortalized nontumorigenic epithelial cell line derived from tissue from a reduction mammoplasty, was grown in equal parts of DMEM (with 4.5 g/l glucose and without L-glutamine) and HyQ Ham's F-12 supplemented with 5% horse serum (Biosource, Camarillo, California, USA), 10 μg/ml of insulin, 500 ng/ml of hydrocortisone, 100 ng/ml of cholera toxin and 20 ng/ml of epidermal growth factor in a 5% CO_2 atmosphere. Human prostate cancer cell lines DU145 [19] and LNCaP [20] were grown in Roswell Park Memorial Institute medium 1640 (RPMI 1640), 10% fetal calf serum, penicillin

(100 units/ml) and streptomycin (100 μg/ml). Human prostate cancer cell line PC3 [19] was grown in DMEM/F12 (Invitrogen Corporation, Grand Island, New York, USA), L-glutamine (2 mmol/l), 5% fetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). For proliferation assays, cells were seeded in 24-well plates and PBS or TE was added at time 0. Forty-eight hours later, cells were trypsinized and the viable cell number was determined by trypan blue staining or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Tumorigenicity and metastasis assays

PBS (150 or 200 μl) or TE (150 or 200 μl) was injected intraperitoneally into syngeneic Balb/cByJ female mice on days 1–4. On day 4, 1×10^5 line, 66.1 or 410.4 tumor cells were injected into the lateral tail vein. Treatment with PBS or TE continued (200 μl) daily for an additional 6 days. Between days 16 and 21 after tumor cell injections, when control animals became moribund, mice were euthanized and surface lung tumor colonies were counted under a dissecting microscope [21]. To determine the effect of TE on established tumors, 5×10^5 line, 410.4 tumor cells were injected subcutaneously proximal to the mammary gland of Balb/cByJ female mice. Two hundred micro-liters of either PBS or TE was injected intraperitoneally daily for 18 days, starting on day 5 when tumors became palpable. Tumor growth was monitored by caliper measurement as described in [12]. When tumors achieved an average diameter of 18 mm, or earlier if animals appeared moribund, mice were euthanized individually and soft tissues were examined for spontaneous metastasis.

Prostaglandin assays

Cells (410.4 and 66.1) were seeded in 12 ml of growth media. TE was added at a final concentration of 6.25–100 μg protein/ml. Control dishes contained PBS. Twenty-four hours later, media were collected and the PGE_2 level was determined by EIA (Cayman Chemicals, Ann Arbor, Michigan, USA).

Real-time PCR

Total RNA was extracted from cultured cells using TRIzol (Invitrogen), and single-stranded cDNA was generated from 1 μg of total RNA by means of reverse transcription using qScript cDNA SuperMix (Quanta BiosciencesInc., Gaithersburg, Maryland, USA). Quantitative PCR amplification was performed using iQ SYBR Green Supermix iQ (Bio-Rad, Hercules, California, USA) and the following gene-specific primers: 5′-CCGAGGTG TATG TAT GAGTGTG-3′ (sense) and 5′-TGAAGTGGG TAAGTATGTAGTGC-3′ (anti-sense) for mouse COX-2, 5′-GGTGACAACTGGAGGGAGGAG-3′ (sense) and 5′-TC TGGGAGTGG ATGGATGTGC-3′ (anti-sense) for COX-1, and 5′-GCCTTCC GT GTTCCTACC-3′ (sense) and 5′-GCCTGCTTCACCACCTTC-3′ (anti-sense) for mouse GAPDH. The threshold cycle (C_t) values obtained were processed for further calculations according to the comparative C_t method. Expression levels of target genes were normalized to the housekeeping gene GAPDH, yielding the ΔCt value. Finally, the gene expression level was calculated as $2^{-\Delta Ct}$, yielding the final value that is normalized to the housekeeping gene.

Migration assay

Calcein AM-labeled tumor cells (Molecular Probes, Eugene, Oregon, USA) were pretreated with TE at a final concentration of 6.25–50 μg/ml and were placed in the upper well of Millicell tissue culture plate well inserts (Millipore Ireland Ltd, Tullagreen, Carrigtwohill, Co Cork, Ireland). Two percentage of FBS was placed in the bottom chamber. To some wells, base medium or as a positive control 2% FBS was placed in the bottom chamber and untreated cells were placed in the upper well. Twenty-four hours later, migration was assessed after removing nonmigrating cells from the upper chamber using a DTX 880 multimode detector measuring fluorescence at 485 nm. Results expressed as optical density (mean ± standard error) of triplicate wells.

Statistical analysis

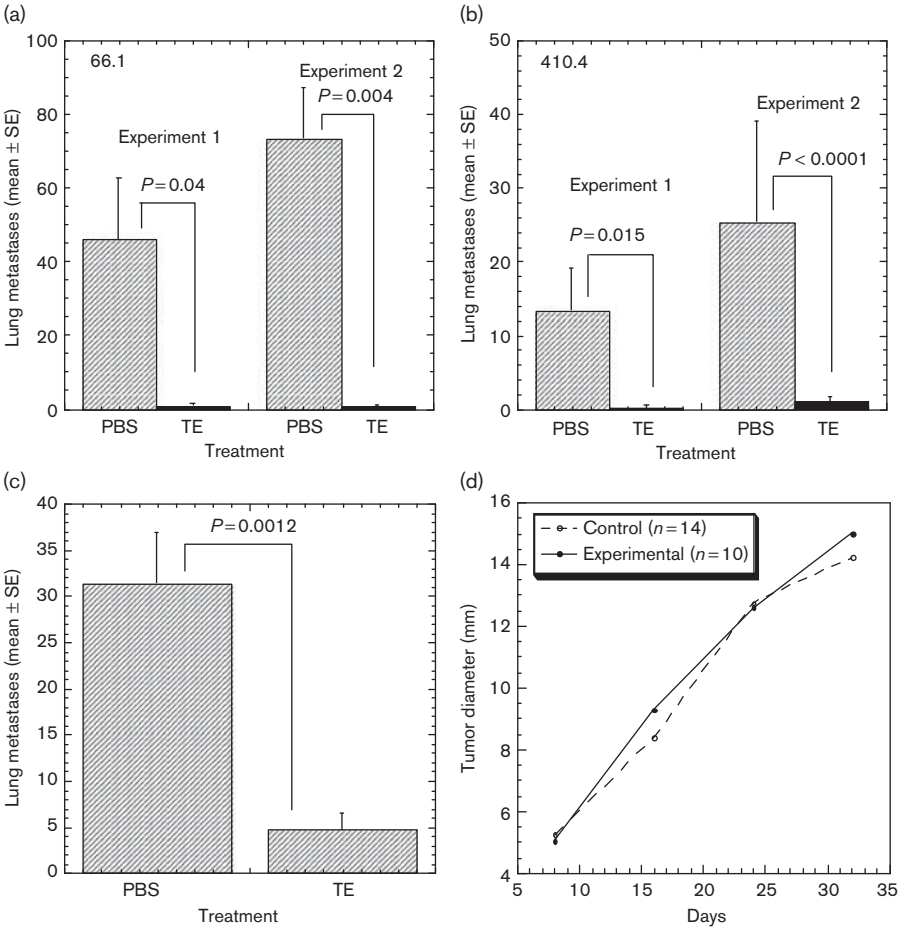
Data were summarized using descriptive statistics means and standard errors, medians and ranges. Depending on the data distribution, Student's *t*-test, or its nonparametric alternative, the Wilcoxon test, was used to compare the distribution of metastases between treatment groups. All statistical tests were exact and carried out at the two-sided 0.05 level of significance.

Results

Taro extract inhibits lung colonization

We first examined the effect of stock TE on lung-colonizing ability of highly metastatic murine breast cancer cell lines 66.1 and 410.4; the latter forms colonies in both the lung and the heart after an intravenous administration. PBS (control) or TE was injected intraperitoneally daily into syngeneic Balb/cByJ female mice on days 1–10. On day 4, either 66.1 or 410.4 tumor cells were injected into the lateral tail vein. Between days 16 and 21 after tumor cell injections, when control animals became moribund, mice were euthanized and surface lung tumor colonies were counted. Figure 1a and b summarize the effect of TE on lung colonization by 66.1 and 410.4 cell lines, respectively, in two independent experiments, carried out with two independently prepared extracts. In all experiments, TE profoundly inhibited the ability of tumor cells to colonize the lungs. TE treatment resulted in a 98–99% reduction in lung tumor colony formation by either tumor cell line. In the case of 410.4, TE also significantly inhibited tumor colonies in the heart. In two experiments, tumor colonies were detected in four of five and five of nine control

Fig. 1



Taro extract (TE) inhibits lung colonization and spontaneous metastasis. The effect of TE on lung-colonizing ability of highly metastatic lines 66.1 (a) and line 410.4 (b) was determined. (a) 150 μl of phosphate-buffered saline (PBS) or TE or (b) 200 μl of PBS or TE was injected intraperitoneally daily for 10 days. On day 4, 1×10^5 line 66.1 or 410.4 tumor cells were injected into the lateral tail vein. On day 21 after tumor cell injection, mice were euthanized and surface lung tumor colonies were counted. (c) 5×10^5 line 410.4 tumor cells were injected subcutaneously proximal to the mammary gland of mice. When the tumor became palpable (day +5), 200 μl of either PBS or TE was injected intraperitoneally daily for the next 18 days. Tumor growth was monitored and when tumors measured 18 mm in average diameter or earlier if animals appeared moribund, mice were euthanized and spontaneous lung metastases were enumerated. (d) The null effect of TE on tumor growth.

mice, whereas none (0/5) and only one of 10 TE-treated mice showed heart involvement (data not shown).

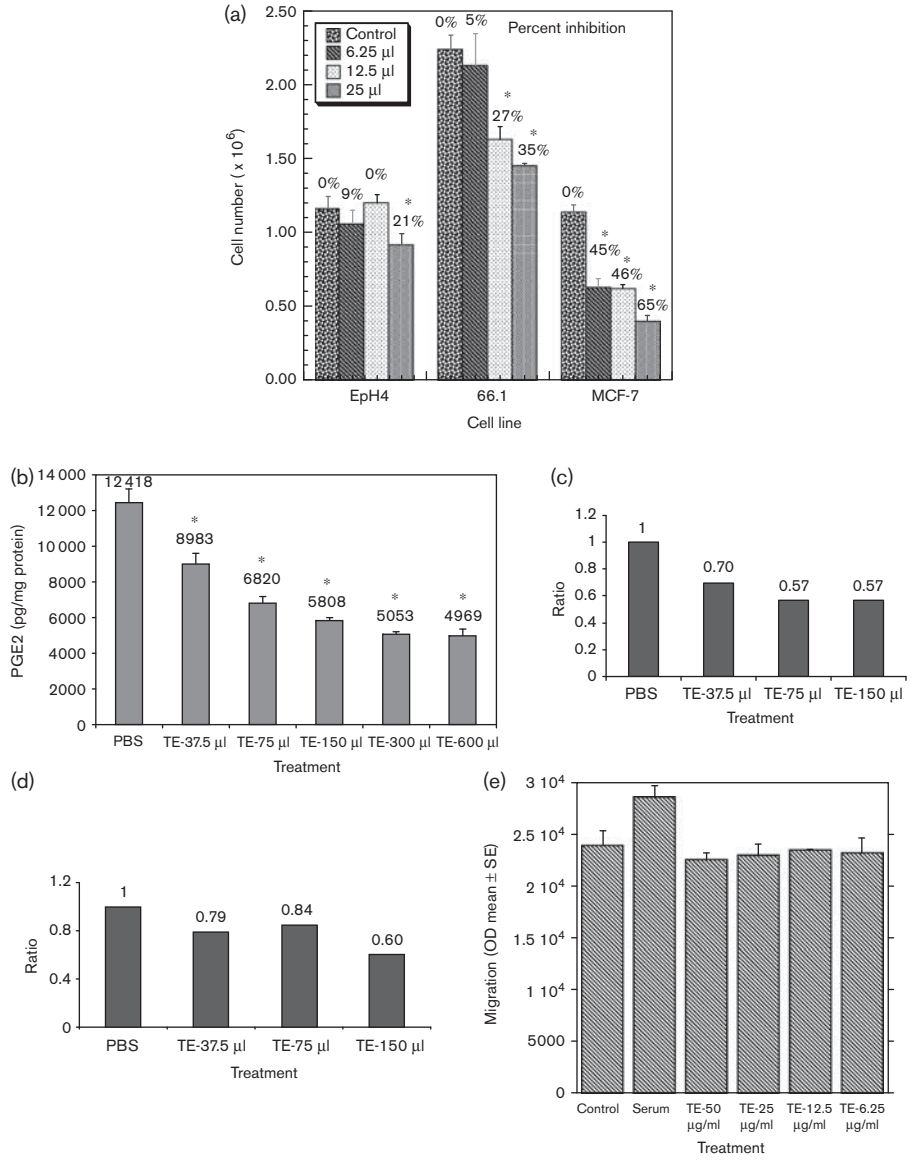
Taro extract inhibits established tumor metastasis

To determine the effect of TE on established tumors and spontaneous metastasis, mice were transplanted subcutaneously with 5×10^5 410.4 cells proximal to the right inguinal mammary gland. Beginning on day 5, when tumors became palpable, 200 μl of either PBS or TE was injected intraperitoneally daily for 18 days. When tumors achieved an average diameter of 18 mm, or earlier if animals appeared moribund, mice were euthanized individually and soft tissues were examined for spontaneous metastasis. Delaying the initiation of TE therapy until tumors are well established still resulted in a significantly reduced number (85% inhibition) of spontaneous lung metastases (Fig. 1c) but had no effect on the size of the locally growing tumors (Fig. 1d).

Effect of taro extract on cell morphology and cell proliferation

To investigate the mechanisms responsible for TE-mediated inhibition of metastasis, we examined the effect of TE on the morphology and proliferation of a panel of murine (66.1, 410.4) and human (MCF-7, MDA-MB-231, MDA-MB-435 and T47D) breast cancer cell lines, human prostate cancer cell lines (DU145, LNCaP, PC3) and immortalized murine mammary (EpH4) and human mammary (MCF10A) epithelial cell lines. TE profoundly affected the morphology of some (66.1, MCF-7, LNCaP) but not all tumor cells, resulting in the retraction of cellular foot processes and cell rounding,

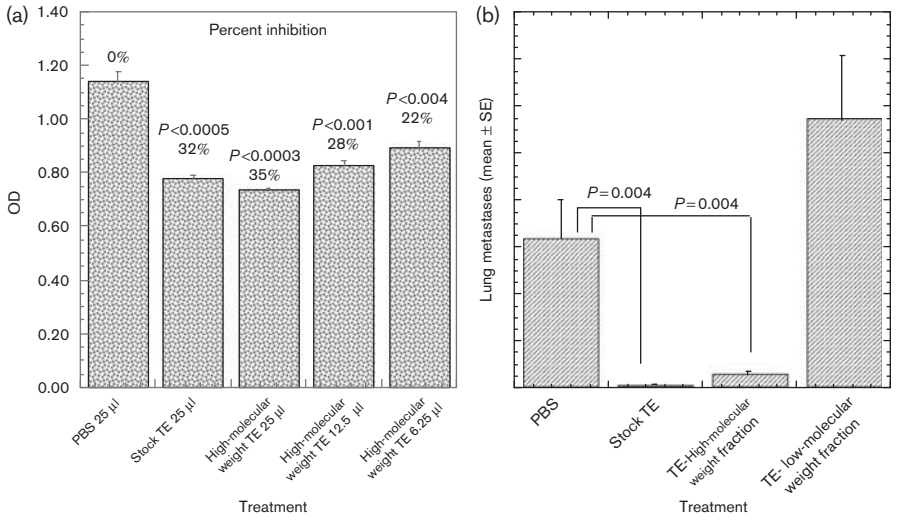
Fig. 2



Taro extract (TE) inhibits cell proliferation, prostaglandin E_2 (PGE_2) synthesis, COX mRNA expression and migration. (a) Line EpH4, 66.1 or MCF-7 cells were seeded at 2.5×10^5 cells/well/1.0 ml media in triplicate in 24-well plates. Phosphate-buffered saline (PBS) or TE (6.25, 12.5, 25 µl) was added. Forty-eight hours later, the cell number was determined. (*$P < 0.05$). (b) 6×10^6 of line 410.4 cells were seeded in 12 ml of media in triplicate. TE from a stock concentration of 2 µg/µl was added to the media in the indicated amounts. Control dishes contained PBS at the highest amount used with TE. Twenty-four hours later, cell-conditioned media were collected and the PGE_2 level was determined by enzyme-linked immunosorbent assay (EIA). Data expressed as pg prostaglandin E_2 (PGE_2)/mg protein as mean ± standard error (SE; *$P < 0.05$). (c and d) 6×10^6 line 410.4 cells were seeded in 12 ml of media and 24 h after plating, TE was added as in (b). Six hours later, RNA was isolated, reverse transcribed and amplified using a primer specific for COX-2 (c) and COX-1 (d). (e) Line 410.4 tumor cells, untreated or pretreated with TE (6.25–50 µg/ml) for 1 h, were placed in the upper chamber of a modified Boyden assay. Media or media containing 2% serum were added to the lower chamber. Cells traversing the membrane were quantified 24 h later. OD, optical density.

whereas the appearance of immortalized normal mammary epithelial cell lines MCF10A (human) and EpH4 (murine) remained unchanged even at the highest concentration of TE (data not shown). TE significantly inhibited the proliferation of 66.1, MCF-7, 410.4, MDA-MB-231 and MCF10A cells in a dose-dependent manner. At the highest TE concentrations (50 µg/ml) line 66.1 and MCF-7 cells were reduced by 35 and 65% (Fig. 2a). EpH4 cell number was reduced only at the highest TE concentration used (Fig. 2a). Similarly, the growth of lines 410.4, MDA-MB-231 and MCF10A was reduced by 24, 26 and 31% (data not shown). The proliferation of the other breast cancer cell lines including MDA-MB-468 or T47D cells was not affected. The observation that some, but

Fig. 3



Effect of high and low-molecular weight taro extract (TE) on cell proliferation and metastasis. (a) Line 66.1 cells were seeded in 24-well plates and PBS or high-molecular weight fraction of TE was added at 6.25, 12.5 and 25 µl doses at time 0. Forty-eight hours later, cell number was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. (b) 100 µl high-molecular weight fraction, 200 µl of phosphate-buffered saline (PBS), stock TE or low molecular weight fraction of TE was injected intraperitoneally for 10 days. On day 4 of treatment, 2×10^5 line 66.1 tumor cells were injected intravenously. On day 19 after tumor cell injections, mice were euthanized and surface lung and heart tumor colonies were counted. *P* values by the Wilcoxon exact two-sided test at the 0.05 level of significance were analysed. OD, optical density.

not all, cells were adversely affected by TE suggests that the antiproliferative effects of TE are not likely to be a result of nonspecific toxicity. In addition, the antiproliferative effect of TE is reversible with time. Polymyxin B treatment of TE did not reduce the antiproliferative activity, ruling out endotoxin contamination as a likely explanation for these observations. We have also determined that the antiproliferative activity of TE is markedly reduced by boiling or by TCA precipitation of the extract before addition to cells (not shown).

Taro extract inhibits cyclooxygenase activity

An increased PGE_2 production is a very common feature in human malignancies. PGE_2 levels are positively correlated with increased tumorigenic and metastatic potential [10]. We examined the effect of TE on PGE_2 production by 410.4 and 66.1 cells. TE inhibits PGE_2 production significantly by 410.4 cells in a dose-dependent manner (Fig. 2b) and in 66.1 cells (data not shown). At the highest concentration of TE, PGE_2 release was reduced by 63% compared with PBS-treated control cells. PGE_2 production is mediated by two COX isoforms. We treated 410.4 cells with TE for 6 h, mRNA was harvested and the levels of COX-2 and COX-1 mRNA were determined by quantitative PCR. TE treatment inhibited the expression of COX-2 mRNA and, to a lesser extent, COX-1 (Fig. 2c and d).

Taro extract inhibits cell migration

Tumor cell migration is an important aspect of metastasis. We asked whether TE treatment modulates migratory ability. Tumor cells migrate in response to FBS. TE completely inhibited tumor cell migration in response to serum at all concentrations examined (Fig. 2e).

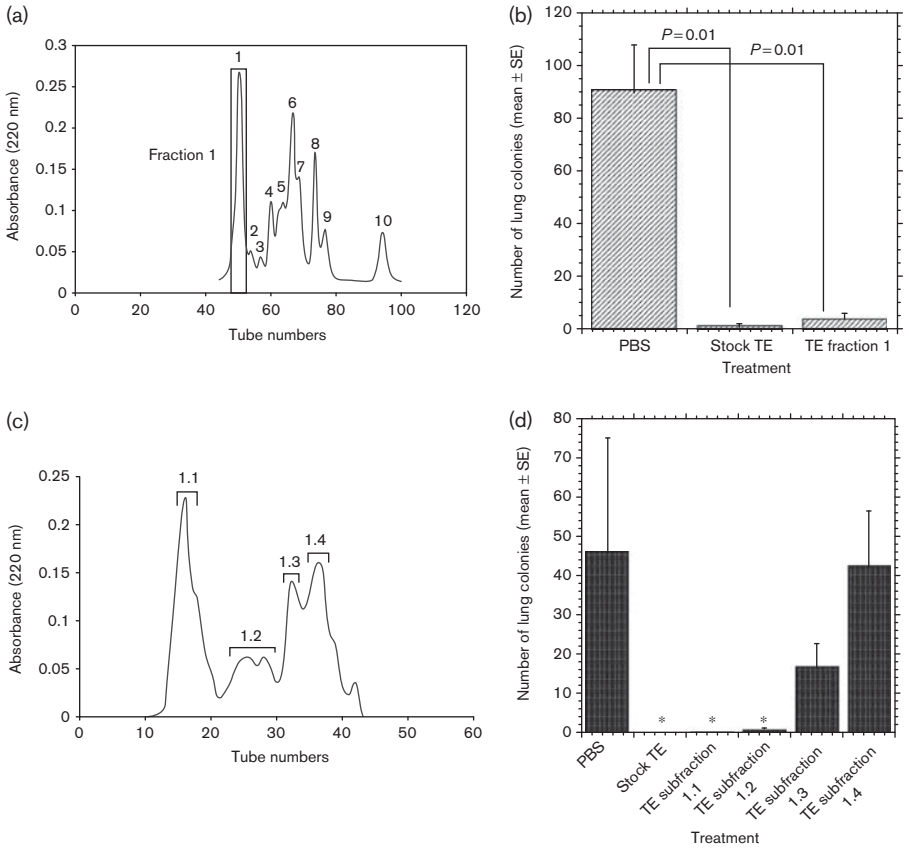
Effect of high-molecular weight and low-molecular weight fractions of taro extract on cell proliferation

To identify the active component of TE, we compared the antiproliferative activity of high-molecular weight and low-molecular weight subfractions to TE at 6.25, 12.5 and 25 µl doses. A significant reduction of cell number was observed in response to treatment with stock TE (32%) or the high-molecular weight fraction (22, 28 and 35%, respectively; Fig. 3a). No significant effect on cell number was observed in the presence of the low-molecular weight fraction at any concentration (data not shown). Thus, the inhibitory effect of TE on cell growth is mainly because of the high-molecular weight fraction.

High-molecular weight fraction inhibits lung colonization

To determine whether antimetastatic activity is also present in the high-molecular weight fraction of TE, we compared the effect of stock TE, high-molecular weight fraction of TE and low-molecular weight fraction of TE on lung colonization. As high-molecular weight compounds were concentrated in the upper fraction (protein concentration is twice the stock), 100 µl of high-molecular weight fraction, 200 µl of PBS, stock TE or low-molecular weight fraction of TE was injected daily per mouse intraperitoneally for 4 days. On day 4, 2×10^5

Fig. 4



Fraction profile from size exclusion (SEC) and anion-exchange chromatography and effect on lung colonization. (a) Stock taro extract (TE) was analyzed by preparative SEC and 10 fractions were obtained. (b) Phosphate-buffered saline (PBS) (control), 400 μg of TE stock or 20 μg of fraction 1 from SEC injected intraperitoneally for 10 days. On day 4, 1×10^5 line 66.1 tumor cells were injected intravenously. On day 18 after tumor cell injections, mice were euthanized and surface lung tumor colonies were enumerated. (c) Fraction 1 further purified by anion-exchange chromatography. (d) Four subfractions from anion-exchange chromatography were assessed in the lung colonization assay using line 66.1. PBS, 400 μg of TE stock or 20 μg of subfraction 1.1, 1.2, 1.3 or 1.4 injected intraperitoneally and treatment continued as in (b). *P* values by the Wilcoxon exact two-sided test at the 0.05 level of significance. (*$P < 0.0001$).

line 66.1 tumor cells were injected into the lateral tail vein. The PBS or TE treatments were continued intraperitoneally daily for an additional 6 days. On day 19 after tumor cell injections, mice were euthanized, surface lung tumor colonies were counted and the presence of heart metastases was noted (Fig. 3b). The results confirm our previous observation that stock TE significantly inhibits the ability of tumor cells to colonize the lungs compared with the vehicle-treated control mice. Similarly, the high-molecular weight fraction of TE also significantly inhibited lung colonization but the antimetastatic effect was completely absent from the low-molecular weight fraction. Although three of seven control animals and four of eight mice treated with a low-molecular weight fraction developed tumor colonies in the heart, only two of 10 and zero of 10 mice treated with stock or a high-molecular weight fraction, respectively, developed heart metastases.

Fraction profile by size exclusion chromatography

We established a protein purification scheme to identify the active component(s). Using SEC, we obtained 10 fractions from Stock TE (Fig. 4a). All 10 fractions were tested for antiproliferative activity *in vitro* using 66.1 tumor cells but only fraction 1, approximately 30 kDa from SEC (calibrated using globular proteins), showed modest antiproliferative activity (data not shown). We evaluated the ability of fraction 1 to inhibit the lung-colonizing ability of 66.1 cells in comparison with stock TE or PBS. Despite the modest effects on proliferation, fraction 1 showed potent antimetastatic activity comparable with stock TE (Fig. 4b). Fraction 1 was further purified by anion-exchange column chromatography. Four subfractions obtained from this step (Fig. 4c) were assessed in the lung colonization assay and compared with the antimetastatic activity of stock TE (Fig. 4d). The majority of the antimetastatic activity was recovered

from the first and the second subfraction peaks. Activity gradually declines in the third peak and is almost lost in the fourth peak.

Fraction profile from reversed-phase liquid chromatography

To determine the purity of each subfraction, reversed-phase chromatography was used (Fig. 5a, b, c, and d). Only one protein peak (at 23.4 min) was eluted from subfraction 1.1 (Fig. 5a), suggesting that the fraction having antimetastatic activity is in an almost pure form. When other subfractions were analyzed on RPLC, multiple peaks including subfraction 1.1 (at 23.4 min) were eluted as contaminants, in decreasing order (Fig. 5b, c, and d). This probably explains why subfraction 1.2 also shows significant antimetastatic activity but not subfraction 1.4.

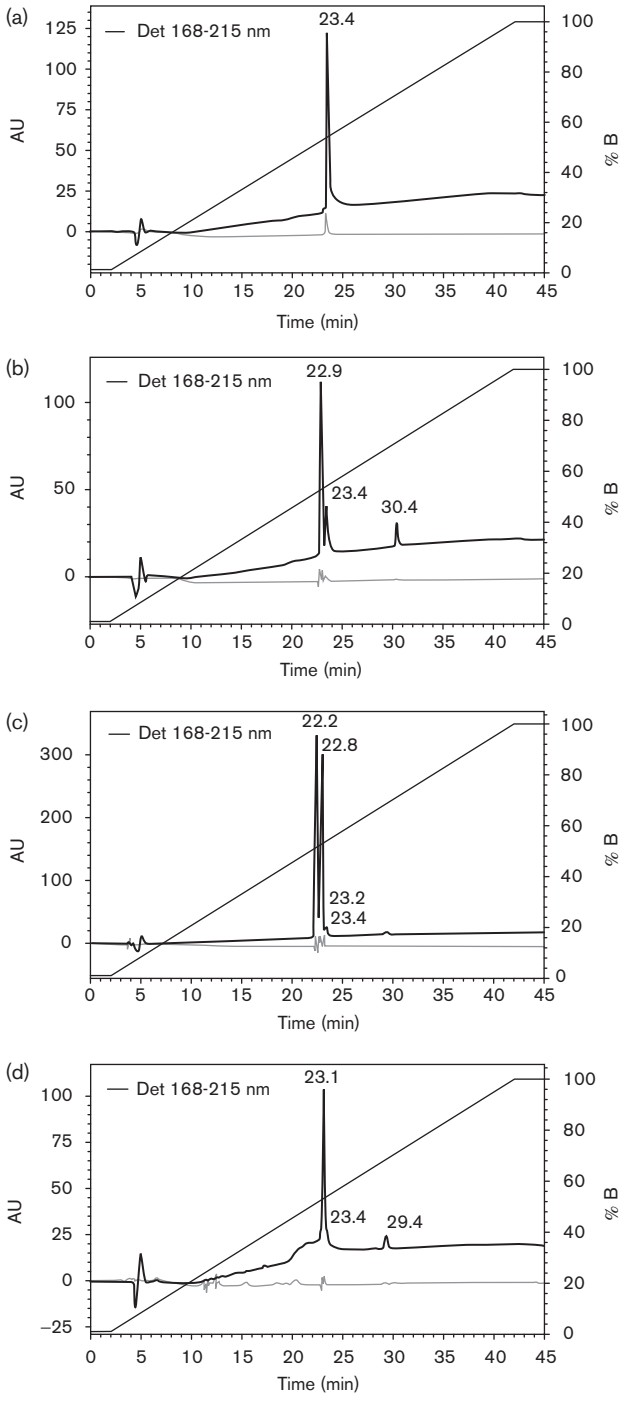
Active component(s) consists of two protein fragments of 12 and 13 kDa

When the active component(s) was analyzed from subfraction 1.1 by SDS-PAGE (Fig. 6a), two protein bands were seen with sizes of approximately 12 and 13 kD. As the native size of the active component(s) determined by the sizing column is around 30 kDa, the active component(s) likely consist of the two fragments seen in SDS-PAGE at a 1 : 1 ratio. This hypothesis is further supported by the analyses of the N-terminal sequences of the active components (Table 1). When the pooled active components were subjected to automated Edman degradation, two major phenylthiohydantoin-amino acid signals in each cycle were observed at an almost equimolar ratio (Table 1). Taken together, the active component(s) appears to be a 25-kDa protein that contains two subunits with sizes of 12 and 13 kDa.

Active component(s) is highly related to taro storage protein, tarin and taro lectin

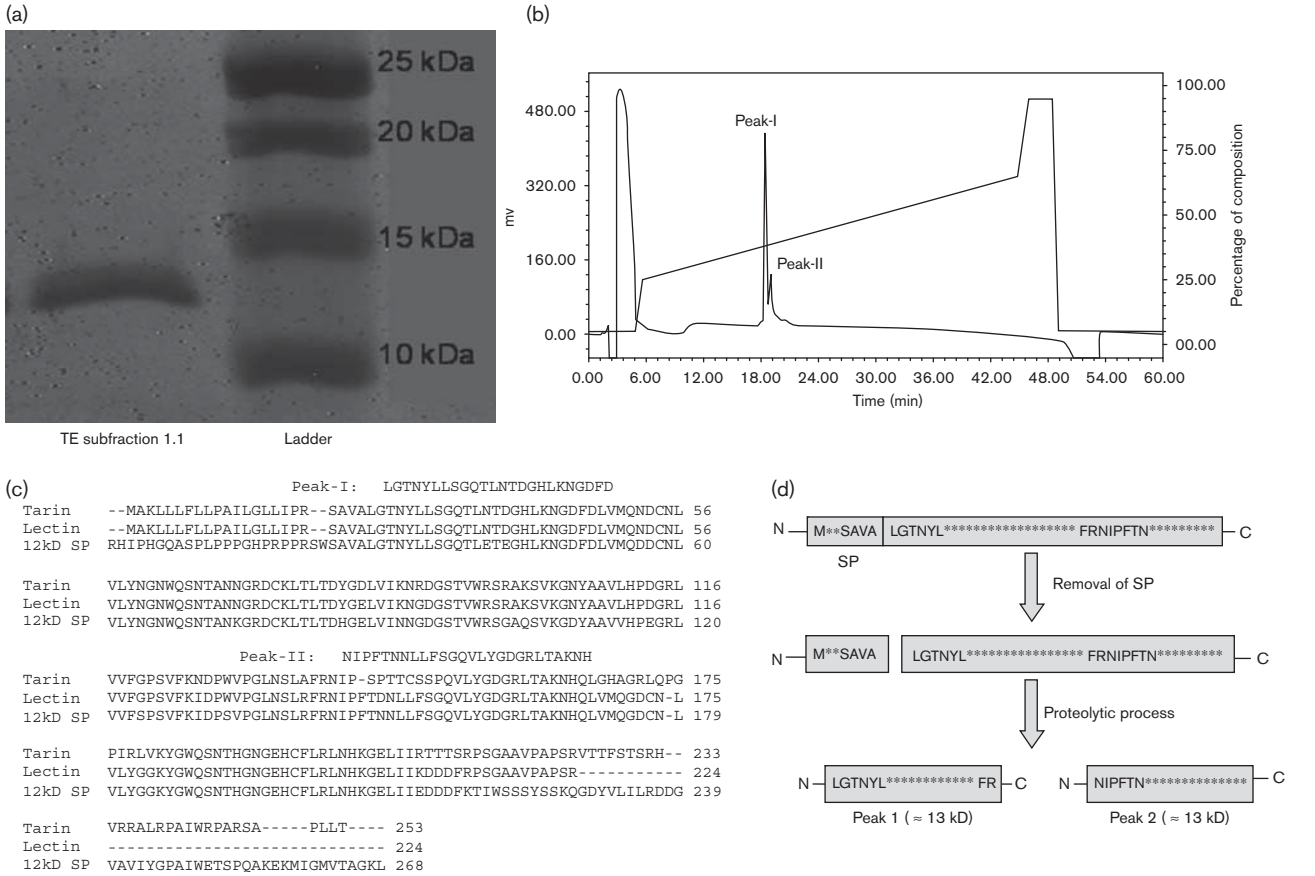
To obtain the N-terminal sequences of the two fragments, the active component(s) pooled from Poros HQ/20 column were purified further by rpHPLC (Fig. 6b). Two partially resolved peaks were obtained. The amino acid sequence for peak-I (Fig. 6c) was determined to be **LGTNYLLSGQTLNTDGHLKNGDFD** and the sequence of the second peak in the fractions was deduced to be **NIPFTNNLLFSGQVLYGDGRLTAKNH** by subtracting the sequence obtained for peak-I from the data obtained previously with both sequences (Table 1). A BLAST similarity search of the sequence data against the nr database reveals that the active component(s) is highly related to three taro proteins, including the taro 12-kDa storage protein (accession number BAA03722) [22], tarin (accession number CAA53717) [23] and the taro lectin (accession number ABQ32294). The 24 N-terminal amino acid sequence of the protein in peak-I is identical to both taro lectin and tarin, which appear to be distinct gene products with identical N-terminal amino acid sequences that significantly diverge after amino acid 142. The N-terminal sequence of the peak-I protein is also nearly identical to a third taro protein, 12-kDa storage protein, with

Fig. 5



Fraction profile from reversed-phase liquid chromatography. Each subfraction from anion-exchange column was analyzed by reversed-phase chromatography of subfractions 1.1 (a), 1.2 (b), 1.3 (c) and 1.4 (d). The apparent peak of interest is at 23.4 min. AU, absorbance units.

Fig. 6



Protein identification. (a) SDS-polyacrylamide gel electrophoresis of subfraction 1.1 under reducing conditions. (b) Additional purification of isolated proteins by reversed-phase high-performance liquid chromatography. (c) The amino acid sequences of the three taro proteins (tarin, lectin and 12-kDa storage protein) and the comparison of amino acid sequence location of peaks 1 and 2 with these three proteins. (d) A schematic representation of the proposed posttranslational processing of the active compound. The three taro proteins, 12-kDa storage protein, tarin and lectin, appear to undergo an identical two-step maturation process to generate mature forms with two fragments: the signal peptides (SP) are removed by a cleavage at SAVA-LGTN, followed by a second proteolytic cleavage at FR-NIP to generate two fragments.

Table 1 Phenylthiohydantoin–amino acid yields from IEX fractions

Cycle	Called residues	Yield (pmoles)
1	N	54.3
	L	66.3
2	G	53.1
	I	58.7
3	T	54.9
	P	50.6
4	N	41.5
	F	49.3

Automated Edman degradation was performed on an Applied Biosystems 494 HT. Mass spectrometric analysis was performed on a Shimadzu Axima CFR Plus using sinapinic acid as the matrix. Proteins were identified by a similarity search of the nr database using the BLASTP 2.2.24+ algorithm by the National Center of Biotechnology Information web interface.

differences in only two amino acid residues. Interestingly, the 26 amino acid sequence identified in the peak-II protein is identical to that of the 12-kDa storage protein. The N-terminal sequence of the peak-II protein is also highly related to tarin with 72% identity (18 of 25 amino acid residues) and taro lectin with 96% identity (24 of 25 amino acid residues). Examination of the original sequence data from the ion-exchange fractions showed that the yield of phenylthiohydantoin–glutamic acid in cycles 13 and 15 was close to the background level, indicating that the N-terminal polypeptide chain (residues 28–143) of 12-kDa storage protein was not present or was below the level of detection. The three taro proteins appear to undergo an identical two-step maturation process to generate mature forms with two fragments: the signal peptides are removed by a cleavage at SAVA-LGTN, followed by a second proteolytic cleavage at FR-NIP to generate two fragments (Fig. 6d). As the active component(s) contains two fragments with N-terminal sequences matching to the two cleavage sites, respectively, the active component(s) resembles the taro proteins in terms of the maturation process as summarized (Fig. 6d). All three proteins,

Table 2 Effect of taro extract on body and organ weight

	Body weight gain (mg)	Spleen (mg)	Liver (mg)	Kidney (mg)	Heart (mg)	Lung (mg)
PBS ($n=3$)	73 ± 10	94 ± 6	1000 ± 45	293 ± 7	137 ± 9	158 ± 4
Subfraction 1.1 ($n=3$)	89 ± 17	121 ± 9	1159 ± 67	293 ± 10	134 ± 5	163 ± 12
Stock TE ($n=3$)	78 ± 17	183 ± 12	1553 ± 116	345 ± 22	144 ± 10	206 ± 16
		$P=0.006$	$P=0.03$			

Normal mice were treated intraperitoneally with PBS, 400 μg of TE or 20 μg of subfraction 1.1 for 4 days. On day 5, mice were euthanized and organ wet weight was determined. Body weight was assessed on days 1 and 5 and the difference was recorded as body weight gain. Organ weights were compared with PBS-treated mice and *P* values were determined using Student's *t*-test. Values are: mean ± SE.
PBS, phosphate-buffered saline; SE, standard error; TE, taro extract.

12-kDa storage protein, tarin and taro lectin, contain a carbohydrate-binding domain.

Effect of stock taro extract or subfraction 1.1 treatment on weight

To examine the potential toxicity of TE, normal mice were treated intraperitoneally with PBS, 400 μg of stock TE or 20 μg of subfraction 1.1 for 4 days. On day 5, mice were euthanized and organ wet weight was determined. Body weight was assessed on days 1 and 5 and the difference was recorded as body weight gain (Table 2). The gross appearance of the animals was not different among the control, stock TE or subfraction 1.1-treated mice. Spleens and livers were significantly heavier in TE-treated mice, whereas subfraction 1.1-treated mice showed no differences in any organ weight compared with PBS-treated control mice. To evaluate the long-term effects on organ weight, three mice from PBS-treated and TE-treated groups were euthanized on day 36 (32 days after the treatment ended). Spleens from control mice weighed 124 ± 2 versus 127 ± 1 mg in TE-treated mice. Thus, the TE-induced splenomegaly is reversible when the treatment is stopped; subfraction 1.1 does not induce spleen enlargement.

Discussion

Taro (*Colocasia esculenta*) is a tropical plant that is a major dietary staple in many regions of Asia and Africa. Taro corms are very high in starch and are a good source of dietary fiber. Raw taro contains sodium, carbohydrate, dietary fiber, sugars, protein, vitamins and minerals [24]. Some early studies suggested that poi, a pasty starch made from cooked, mashed corm of the taro plant might be useful for the treatment of allergies, failure-to-thrive in infants and certain gastrointestinal conditions [25]. Fiber derived from taro could adsorb the mutagens 1,8-dinitropyrene [26]. A soluble extract of cooked taro (poi) has previously been shown by Brown *et al.* [13] to inhibit proliferation of the rat YYT colon cancer cell line *in vitro* but the potential anti-cancer and antimetastatic activities of taro have not been investigated *in vivo*. Our study demonstrates for the first time that a water-soluble extract of uncooked taro corm has potent antimetastatic activity in a murine model of metastatic breast cancer. Using two highly metastatic, estrogen receptor, progesterone receptor and Her-2/neu negative murine mammary tumor cell lines, we showed that TE treatment leads to nearly complete ablation of metastasis in a lung colonization model. In a more clinically relevant model, TE treatment was initiated after mammary tumors were established in mice. Significant inhibition of spontaneous metastases to the lung was observed in TE-treated mice. Thus, TE is efficacious in both prevention and therapeutic models.

We sought the relevant mechanisms underlying the therapeutic effect of TE. TE modestly inhibited the proliferation of some murine and human breast and prostate cancer cell lines. These findings are somewhat similar to those of Brown *et al.* [13]. We observed that the antiproliferative effect is reversible and is accompanied by morphologic changes; TE-treated cells were rounded and displayed fewer cellular extensions. Observations that some but not all cell lines are affected by TE argue against a non-selective toxicity. The modest effects on proliferation *in vitro* were not reflected in any demonstrable inhibition of the growth of subcutaneously implanted tumors. Furthermore, fraction 1 showed very little antiproliferative activity but significant antimetastatic activity comparable to stock TE, suggesting that antiproliferative and antimetastatic activities do not occur by the same mechanism. Given the potent antimetastatic activity of TE, we were somewhat surprised that the growth of the mammary gland-implanted tumor was not reduced. It may be that higher doses are required to observe an effect on local growth. It is difficult to obtain sufficient SF-1.1 to determine whether this fraction affects local tumor growth and this question must wait until recombinant proteins can be produced and evaluated. The current data suggest, however, that TE may specifically target mechanisms underlying tumor dissemination. The complete inhibition of tumor cell migration by TE is consistent with this hypothesis.

It is well established that there is a direct positive correlation between elevated level of prostaglandin synthesis and COX-2 expression with increased tumorigenicity and metastasis [8–10]. In this study, we have shown that TE treatment inhibits PGE_2 synthesis and COX-1 and COX-2 enzyme expression *in vitro*. The majority of PGE_2 released by these cells is generated by the COX-2 isoform and is inhibitable with selective COX-2 inhibitors [10]. Future studies will

explore the mechanisms by which TE downregulates COX mRNA.

Further purification of fraction 1 by anion exchange chromatography and RPLC indicates that potent antimetastatic activity occurs because of subfraction 1.1. Subfraction 1.1 from IEX also showed one major peak but SDS PAGE, rpHPLC and Edman degradation analysis of the subfraction 1.1 showed the presence of two peptides. It is possible that the native active protein(s) of similar size and sequence dissociated into two polypeptide chains. Two polypeptides were identified in subfraction 1.1 in nearly equimolar amounts. As Edman degradation requires a free amino terminus for coupling of phenylisothiocyanate, proteins with a chemically blocked N-terminus will be refractory to this sequence analysis. Therefore, the presence of additional proteins with blocked N-terminus cannot be ruled out based on this analysis. However, upon further analysis of subfraction 1.1 by rpHPLC, only two peaks were observed. Peak-I yielded a single amino acid sequence that, upon a BLAST search of the nr database, showed an N-terminal sequence identical to two entries: tarin (accession CAA53717) and lectin (accession ABQ32294). Both of these database entries have identical amino acid sequences until position 142, after which their amino acid sequences begin to diverge significantly towards the C terminus. As additional characterization was not performed, it is not clear whether one or both proteins are present in peak-I.

The sequence of the second major protein present in the IEX fractions was deduced by subtracting the sequence obtained from peak-I from the previous sequence data containing the sequences of both proteins. The remaining sequence, when searched against the same nr database, showed sequence identity with 12-kDa storage protein (accession BAA03722). The 12-kDa storage protein is a mixture of two polypeptide chains (12.5 and 13.9 kDa) and the N-terminal sequence of the sample protein aligned to the 13.9-kDa polypeptide of the 12-kDa storage protein sequence beginning at position 144, which is the N-terminus of the 13.9-kDa polypeptide chain. The N-terminal chain of the 12-kDa storage protein shares a high degree of sequence identity to that of the other mannose-binding lectins identified in this study. The presence of the N-terminal chain can be distinguished from the other two mannose-binding lectins by the phenylthiohydantoin–amino acids observed in cycles 13 and 15. A glutamic acid residue occupies these positions in the 12-kDa storage protein whereas asparagine and aspartic acid residues, respectively, occupy these positions in both tarin and lectin. As there were only background levels of phenylthiohydantoin-glutamic acid in these cycles, it was concluded that the N-terminal chain of 12-kDa storage protein (which belongs to the 12.5-kDa polypeptide chain) was either absent or below the level of detection. This sequence was also identical to that of the above database entry, lectin (accession ABQ32294), beginning at position 140. Considering the results from SEC, IEX, rpHPLC, SDS-PAGE and amino acid sequencing, we conclude that the active compound may be tarin, lectin or 12-kDa storage protein; however, a novel protein cannot be excluded.

Mice treated with TE appeared healthy and showed normal weight gain, with the exception that TE caused splenomegaly, which was fully reversible once treatment was stopped. Stock TE also caused an increase in liver weight. Histologic examination by a clinical pathologist (F. Z.) revealed no tissue abnormalities, with the exception of B cell proliferation in the spleens of TE-treated mice. In contrast, subfraction 1.1 induced no significant increase in whole body or individual organ weight, indicating that the cause of this potential toxicity is absent from subfraction 1.1.

We continue to explore the mechanisms underlying these promising results. Storage proteins have multiple functions in addition to their storage roles [23]. It will be important to determine whether recombinant proteins, based on the predicted taro-related proteins, can replicate these observations. Few treatment options are available for metastatic breast cancer. This study identifies a new class of plant-derived compound(s) with potential antimetastatic activity that may prove useful for the therapy of metastatic disease.

Acknowledgements

This work was supported by Public Health Service Grant RO1 CA120278 to A.M.F. from the National Institutes of Health and Human Services. The authors thank the Biostatistics, Confocal microscopy and Genomics shared services of the University of Maryland Greenebaum Cancer Center and the Protein Analysis Laboratory of the Center for Vascular and Inflammatory Diseases, University of Maryland School of Medicine.

Conflicts of interest

There are no conflicts of interest.

References

1 Fahey JW, Zhang Y, Talalay P. Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. *Proc Natl Acad Sci USA* 1997; **94**:10367–10372.
2 Kelloff GJ, Crowell JA, Steel VE, Lubet RA, Malone WA, Boone CW, *et al.* Progress in cancer chemoprevention: development of diet-derived chemopreventive agents. *J Nutr* 2000; **130**:467S–471S.
3 Liu RH. Health benefits of fruit and vegetables and from additive and synergistic combinations of phytochemicals. *Am J Clin Nutr* 2003; **78**:517s–520s.
4 Surh YJ. Cancer chemoprevention with dietary phytochemicals. *Nat Rev Cancer* 2003; **3**:768–780.
5 Milner JA. Molecular targets for bioactive food components. *J Nutr* 2004; **134**:2492s–2498s.
6 Stan SD, Kar S, Stoner GD, Singh SV. Bioactive food components and cancer risk reduction. *J Cell Biochem* 2008; **104**:339–356.
7 Stoner GD, Chen T, Kresty LA, Aziz RM, Reinemann T. Protection against esophageal cancer in rodents with lyophilized berries: potential mechanism. *Nutr Cancer* 2006; **54**:33–46.

8 Taketo MM. Cyclooxygenase-2 inhibitors in tumorigenasis (part I). *J Natl Cancer Inst* 1998; **90**:1529–1536.
9 Ristimaki A, Sivula A, Lundin M, Salminen T, Haglund C, Joensuu H, *et al.* Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer. *Cancer Res* 2002; **62**:632–635.
10 Kundu N, Yang Q, Dorsey R, Fulton AM. Increased cyclooxygenase-2 (COX-2) expression and activity in a murine model of metastatic breast cancer. *Int J Cancer* 2001; **93**:681–686.
11 Kundu N, Smyth MJ, Samsel L, Fulton AM. Cyclooxygenase inhibitors block cell growth, increase ceramide and inhibit cell cycle. *Breast Cancer Res Treat* 2002; **76**:57–64.
12 Kundu N, Fulton AM. Selective cyclooxygenase (COX)-1 or COX-2 inhibitors control metastatic disease in a murine model of breast cancer. *Cancer Res* 2002; **62**:2343–2346.
13 Brown AC, Reitzenstein JE, Liu J, Jadus MR. The anti cancer effects of poi (Colocasia esculenta) on colonic adenocarcinoma cells *in vitro. Phytother Res* 2005; **19**:767–771.
14 Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, *et al.* Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997; **25**:3389–3402.
15 Miller FR, Miller BE, Heppner GH. Characterization of metastatic heterogeneity among subpopulations of a single mouse mammary tumor: heterogeneity in phenotypic stability. *Inv Metas* 1983; **3**:22–31.
16 Lu Y, Zhou H, Chen W, Zhang Y, Hamburger AW. The ErbB3 binding protein EBP1 regulates ErbB2 protein levels and tamoxifen sensitivity in breast cancer cells. *Breast Cancer Res Treat* 2011; **126**:27–36.
17 Price JE, Polyzos A, Zhang RD, Daniels LM. Tumorigenicity and metastasis of human breast carcinoma cell lines in nude mice. *Cancer Res* 1990; **50**:717–721.
18 Balzer EM, Whipple RA, Cho EH, Matrone MA, Martin SS. Antimitotic chemotherapeutics promote adhesive responses in detached and circulating tumor cells. *Breast Cancer Res Treat* 2010; **121**:65–78.
19 Lee DI, Sumbilla C, Lee M, Natesavelalar C, Klein MG, Ross DD, *et al.* Mechanisms of resistance and adaptation to thapsigargin in androgen-independent prostate cancer PC3 and DU145 cells. *Arch Biochem Biophys* 2007; **464**:19–27.
20 Tang Y, Hamburger AW, Wang L, Khan MA, Hussain A. Androgen deprivation and stem cell markers in prostate cancers. *Clin Exp Pathol* 2010; **3**:128–138.
21 Kundu N, Zhang S, Fulton AM. Sublethal oxidative stress inhibits tumor cell adhesion and enhances experimental metastasis of murine mammary carcinoma. *Clin Exp Metastasis* 1995; **13**:16–22.
22 Hirai M, Nakamura K, Imai T, Sato T. cDNAs encoding for storage proteins in the tubers of taro (*Colocasia esculenta Schott*). *Jpn J Genet* 1993; **68**:229–236.
23 Bezerra IC, Csatro LAB, Neshich G, de Almeida ERP, Grossi de Sa' MF, Mello LV, *et al.* A corm-specific gene encodes tarin, a major globulin of taro (*Colocasia esculenta* L. Schott). *Plant Mol Biol* 1995; **28**:137–144.
24 Huang AS, Titchenal CA, Meilleur BA. Nutrient composition of taro corms and breadfruit. *J Food Comp Anal* 2001; **13**:859–864.
25 Brown AC, Valiere A. The medicinal uses of poi. *Nutr Clin Care* 2004; **7**: 69–74.
26 Ferguson LR, Roberton AM, Mckenzie RJ, Watson ME, Harris PJ. Adsorption of a hydrophobic mutagen to dietary fiber from taro (*Colocasia esculenta*), an important food plant of the South Pacific. *Nutr Cancer* 1992; **17**:85–95.