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Frondoside A inhibits breast cancer metastasis and antagonizes prostaglandin E receptors EP4 and EP2

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Abstract

Frondoside A, derived from the sea cucumber *Cucumaria frondosa* has demonstrable anticancer activity in several models, however, the ability of Frondoside A to affect tumor metastasis has not been reported. Using a syngeneic murine model of metastatic breast cancer, we now show that Frondoside A has potent antimetastatic activity. Frondoside A given i.p. to mice bearing mammary gland implanted mammary tumors, inhibits spontaneous tumor metastasis to the lungs. The elevated Cyclooxygenase -2 activity in many malignancies promotes tumor growth and metastasis by producing high levels of PGE₂ which acts on the prostaglandin E receptors, chiefly EP4 and EP2. We examined the ability of Frondoside A to modulate the functions of these EP receptors. We now show that Frondoside A antagonizes the prostaglandin E receptors EP2 and EP4. ³H-PGE₂ binding to recombinant EP2 or EP4-expressing cells was inhibited by Frondoside A at low μM concentrations. Likewise, EP4 or EP2-linked activation of intracellular cAMP as well as EP4-mediated ERK1/2 activation were also inhibited by Frondoside A. Consistent with the antimetastatic activity observed *in vivo*, migration of tumor cells *in vitro* in response to EP4 or EP2 agonists was also inhibited by Frondoside A. These studies identify a new function for an agent with known antitumor activity, and show that the antimetastatic activity may be due in part to a novel mechanism of action. These studies add to the growing body of evidence that Frondoside A may be a promising new agent with potential to treat cancer and may also represent a potential new modality to antagonize EP4.

Keywords

Frondoside A; prostaglandin EP receptor; EP4; EP2; metastasis

Introduction

Sea cucumber has been used for centuries as a delicacy in Asian cuisine and also as a treatment for diverse conditions [1]. Recently antitumor activities have been described for crude fractions and isolated single glycosides from sea cucumbers [2-5]. Frondoside A, a triterpenoid glycoside isolated from the sea cucumber *Cucumaria frondosa* inhibited proliferation and induced apoptosis of pancreatic cancer cell lines as well as growth of human pancreatic and breast cancer xenografts [2,3,5]. A parent compound, Frondanol A5,

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also inhibited growth of a human colon cancer cell line and, furthermore, prevented primary colon carcinogenesis in a rat model [4]. Frondoside A inhibits migration and invasion of breast cancer cell line MDA-MB-231 *in vitro*, but the ability of Frondoside A to affect tumor cell metastasis *in vivo* has not been reported. We investigated the ability of Frondoside A to inhibit metastasis in a syngeneic murine model of metastatic breast cancer. Frondoside A has potent immune modulatory activity [6] and we speculated that Frondoside A could affect tumor cell behavior, in part, by modulating the functions of the key inflammatory mediator, prostaglandin E₂ (PGE₂). PGE₂ mediates cellular signaling through binding to a family of four G-protein coupled receptors (EP1, EP2, EP3, EP4, ref. 7). We now report that Frondoside A is a potent antagonist of the prostaglandin E receptors EP4 and EP2 and this activity may contribute to the antimetastasis mechanism of action.

Materials and Methods

Cells

Line 66.1 was derived from a spontaneously occurring mammary adenocarcinoma in a Balb/c/c3H mouse. Line 66.1 is highly tumorigenic and metastatic following s.c. or i.v. injection into syngeneic Balb/cByJ mice. Cells are maintained in DMEM supplemented with 10% FCS (Gemini BioProducts, Inc., Calabasas, CA), 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 1.5 g/L sodium bicarbonate and 0.1 mM nonessential amino acids.

Cell growth assay

Line 66.1 cells were transfected with a plasmid expressing shRNA targeting the murine EP4 gene or control vector (OpenBiosystems, Huntsville, AL) as described previously [8]. EP4 expression levels were determined by RT-PCR and western blotting of cell lysates. Stable clones expressing reduced levels of EP4 were previously established and evaluated for tumorigenic and metastatic properties [8]. For this study, 1×10^5 cells of a clone expressing 75% less EP4 mRNA than the 66.1-vector cells or parental 66.1 cells were plated in 24-well plates and the next day, Frondoside A in PBS was added at final concentrations ranging from 0.01 µM/L to 1.0 µM/L. Twenty-four hours later, cell number was determined in triplicate determinations. Cell cycle analysis was performed by flow cytometry as described previously (9).

Frondoside A

Frondoside A, a mono-sulfated triterpene glycoside, was isolated from the sea cucumber *Cucumaria frondosa* as previously described [6] and is shown in Figure 1.

³H-PGE₂ binding assays

were conducted by MDS Pharma Services (Taipei, Taiwan) and as described in [10]. Human recombinant HEK-293 cells expressing EP2 or human recombinant Chem-1 cells expressing EP4 were used to assess binding parameters of ³H-PGE₂ during a two hour binding period at 25° C. The ability of Frondoside A (1-40 µg/ml) to inhibit binding of ³H-PGE₂ to both cells was determined and plotted as percent inhibition and the IC₅₀ was calculated.

cAMP assay

Cells were pretreated with indomethacin (1.0 µM/L) for 24 hrs and transferred to complete cell culture medium containing IBMX (100 µM, Sigma Chemical Co., St. Louis, MO). Agonists PGE₂ (5 µM/L), the EP4 agonist PGE₁-OH (5 µM/L), the EP2 agonist butaprost (1 µM/L), the EP2 antagonist AH6809 (1 µM/L), the EP4 antagonist AH23848 (5 µM/L) or Frondoside A (0.1-5.0 µM/L) were added to cells and incubated for 15 min, after which media was aspirated and cell lysates prepared and intracellular cAMP levels determined as

per manufacturer's instructions using the cAMP biotrak EIA system (Cayman Chemicals, Ann Arbor, MI).

Erk1/2 activation assay

Cells were pretreated with DMSO or Frondoside A, then stimulated with PGE₂ or PGE₁-OH (5 μ M/L) for 15 minutes. Cell lysates were immunoblotted with antibody to ERK or phosphorylated-ERK (Cell Signaling, Danvers, MA) and appropriate secondary antibodies conjugated to horseradish peroxidase (KPL, Gaithersburg, MD) and immunoreactivity detected by chemiluminescence (Thermo Scientific, Rockford, IL). Band intensities analyzed using ImageJ and normalization of phosphor-ERK to total ERK proteins.

Mice

Balb/cByJ female mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were housed, cared for, and used in strict accordance with the U.S. Department of Agriculture regulations and the NIH Health Guide for the Care and Use of Laboratory Animals. The University of Maryland School of Medicine Animal Facility is fully accredited by the American Association for the Accreditation of Laboratory Animal Care.

In Vivo Studies

Local tumor growth and spontaneous metastases were evaluated by injecting 5×10^5 tumor cells s.c. proximal to the right abdominal mammary gland of syngeneic female mice (10 mice/group). Tumor diameters were measured with a caliper twice weekly and mice were euthanized on an individual basis when the s.c. tumor measured 18 mm in average diameter or earlier if moribund. The lungs were weighed and surface tumor colonies were quantified in a blinded fashion under a dissecting microscope. Lung colonization was evaluated by injecting 1×10^5 viable tumor cells i.v. into the lateral tail vein of mice. Tumor cells were cultured in the presence of Frondoside A (0.1 or 1.0 μ M/L for 30 minutes) or 5.0 μ M/L for 15 minutes, washed and injected into mice. All mice were euthanized on day 18-22 posttransplantation or earlier if moribund. Lungs were examined for surface tumor colonies.

Migration assay

Tumor cell migration in response to prostaglandins was assessed as previously described [11]. Calcein AM-labeled tumor cells were placed in the upper well of modified Boyden chambers containing nucleopore polycarbonate membranes (8 μ m) coated with a mixture of collagen I and fibronectin. PGE₂ (5 μ M/L), PGE₁-OH (5 μ M/L) or FBS (2%) and Frondoside A (0.1, 1.0 μ M/L) were placed in the bottom chamber. Migration was assessed 24 hours later after removing non-migrating cells from the upper chamber, using a Cytofluor 4000 plate reader measuring fluorescence at 485 nm. Results expressed as the ratio of cells migrating in response to ligand (triplicate wells) relative to basal migration in the control wells in the absence of stimulant.

Statistical Methods

For animal studies, the nonparametric Wilcoxon test was applied to assess plausible differences in the distribution of number of lung metastases between the pre-specified experimental groups. All tests were exact, two-sided and done at the 0.05 level of significance. The Student's t-test was used to compare responses in proliferation, migration and cAMP studies.

Results

Antitumor activities of Frondoside A have been described [2-5] but the ability of Frondoside A to affect tumor metastasis *in vivo* has not been reported. To determine if Frondoside A can modulate tumor dissemination, we employed a well-characterized syngeneic murine model of metastatic breast cancer. To determine the direct effects of Frondoside A on tumor cells, in the absence of host effects, we pretreated line 66.1 mammary tumor cells with Frondoside A, washed the cells and injected 1×10^5 viable cells i.v. into syngeneic Balb/cByJ female mice. Three weeks later, mice were euthanized and number of lung tumor colonies was determined. Pretreatment of 66.1 cells with Frondoside A at $5 \mu\text{M/L}$, prior to injection into mice, reduced lung tumor colony formation by 45% (Figure 2A). In a separate experiment, using two lower concentrations, Frondoside A at $1 \mu\text{M/L}$ also markedly reduced the number of lung tumor colonies (Figure 2B) but exposure to $0.1 \mu\text{M/L}$ Frondoside A was without effect.

To evaluate Frondoside A in a more clinically relevant model, 5×10^5 line 66.1 tumor cells were implanted subcutaneously proximal to the right abdominal mammary gland of mice. Frondoside A at $10 \mu\text{g/kg}$ or $50 \mu\text{g/kg}$ was administered i.p. beginning on day 0 and continuing daily for 10 days. Tumors were measured twice weekly by caliper and when tumors achieved an average diameter of 18 mm, individual mice were euthanized and lung metastases were quantified. Frondoside A treatment at these dose levels had no impact on the size of the mammary gland-implanted tumor (not shown) but, nevertheless, significantly reduced the number of spontaneous metastases to the lungs in mice treated with the higher dose of Frondoside A (Figure 2C). A reduction of spontaneous metastases in mice treated with the lower dose of $10 \mu\text{g/kg}$ was also observed and was marginally statistically significant ($p=0.06$).

Earlier studies showed that Frondoside A had immunomodulatory properties [6, and unpublished]. We hypothesized that Frondoside A might intersect with the proinflammatory cyclooxygenase pathway that significantly contributes to tumorigenic and metastatic behavior in this model [12,13] and in clinical breast cancer [14]. The primary COX-2 product produced in tumors is prostaglandin E_2 which mediates cellular effects by binding to four EP receptors (EP1-EP4). We determined the ability of Frondoside A to inhibit ^3H -PGE₂ binding to recombinant EP receptor-positive cells. For these studies, human HEK cells, transduced to express EP2, or Chem-1 cells expressing EP4 were used in ^3H -PGE₂ binding studies [10]. Based on historical controls, we predicted that EP4 would bind ^3H -PGE₂ with a higher affinity than would the EP2 receptor and that was confirmed (Table 1). The calculated binding affinity for EP4 was $K_d = 0.69 \text{ nM}$ ($B_{\text{max}} = 4.3 \text{ pmole/mg protein}$) vs a K_d of 3.1 nM ($B_{\text{max}} = 1.4 \text{ pmole/mg protein}$) for EP2-expressing HEK cells confirming that these cells bind ^3H -PGE₂ with the expected affinities for EP4 and EP2, respectively. We asked if Frondoside A inhibits binding of ^3H -PGE₂ to EP4-positive cells. Frondoside A inhibits binding of ^3H -PGE₂ to EP4-positive cells with an IC_{50} of approximately $3.7 \mu\text{M}$ (Table 1). Frondoside A also inhibited ^3H -PGE₂ binding to EP2-positive cells with an IC_{50} of $16.5 \mu\text{M}$. Thus, Frondoside can antagonize binding of PGE₂ to both EP2 and EP4 receptors but with a ~4.5-fold higher activity for the EP4 receptor.

To determine if Frondoside A can *functionally* antagonize EP2 and/or EP4, we assessed the ability of Frondoside A to inhibit second messengers that are coupled to EP2 and EP4. It is known that both EP2 and EP4 are coupled to adenylyl cyclase activation on both normal [7] and malignant cells [8] resulting in elevations in intracellular cAMP. For the current studies, we asked if Frondoside A could inhibit EP-coupled cAMP activation in murine breast cancer cells in response to PGE₂ (which binds all four EP receptors) or in response to specific EP4 (PGE₁-OH) or EP2 (butaprost) agonists. For comparison, the EP4 antagonist AH23848 and

the EP2/EP1 antagonist, AH6809 were employed. Exposure of 66.1 cells to PGE₂, which binds all EP receptors, induces high levels of intracellular cAMP; in this experiment approximately 3-fold elevation in intracellular cAMP in comparison to vehicle-treated cells was detected (Figure 3A). As expected, the EP4 antagonist AH23848 only partially inhibited this response because PGE₂ will also activate cAMP through EP2. Frondoside A, at concentrations ranging from 0.1 to 5.0 μ M/L, inhibited the induction of cAMP by PGE₂ in a dose-dependent manner consistent with an EP antagonist function. The EP4 selective agonist PGE₁-OH also activates cAMP in these cells and this response is also completely blocked by Frondoside A (Figure 3B). Even the lowest concentration of Frondoside A was effective and was superior to AH23848 in blocking EP4 activation. Butaprost is a specific agonist of EP2 and treatment of 66.1 cells with butaprost also induced increases in cAMP levels (Figure 3C). This response was blocked by the selective EP2 antagonist, AH6809 or by Frondoside A; in this case, only the two higher concentrations of Frondoside A were able to block EP2-mediated cAMP activation. EP4 also mediates ERK1/2 activation [7]. PGE₂ or PGE₁-OH induced ERK1/2 activation by 1.85 or 2.12 fold, respectively (Figure 3D). Frondoside A was able to inhibit ERK1/2 activation induced by either ligand in a dose-dependent manner. Thus, Frondoside A blocks EP4 and, to a lesser extent, EP2-mediated intracellular signaling.

To determine if, like pancreatic cells, growth of mammary tumor cells is inhibited by Frondoside A, line 66.1 cells were grown in the presence of Frondoside A in a range of concentrations and, 24 hours later, cell number was determined. Frondoside A inhibited growth of 66.1 cells in a dose-dependent manner with 50% inhibition observed at approximately 0.5 μ M/L Frondoside A (Figure 4A). At the same concentration, effects on cell cycle were minimal (data not shown). To determine if the inhibitory effects of Frondoside A on cell number were mediated through EP4, we compared the effect of Frondoside A on proliferation of 66.1 cells stably expressing different levels of EP4. We have previously characterized stable clones of 66.1 expressing shRNA targeting EP4 [8]. For the current study we used an 66.1shEP4 clone which expresses approximately 25% of the EP4 levels detected in 66.1-vector control cells. As observed with parental 66.1 cells, 66.1-vector cells were growth-inhibited by Frondoside A (Figure 4B). Reduced levels of EP4 expression blunted the inhibitory effect of Frondoside A on cell number. For example, at 0.5 μ M/L Frondoside A, growth of 66.1-vector cells was decreased by 51% but by only 18% in 66.1shEP4 cells. These data suggest that the inhibitory effects on cell growth are mediated, in part, through EP4 antagonism.

We hypothesized that the ability of Frondoside A to limit metastasis could be related to underlying effects on tumor cell migration. Frondoside A inhibits migration and invasion of MDA-MB-231 cells in response to serum in a wound healing assay [5]. We have shown previously [11] that PGE₂ can stimulate migration of mammary tumor cells across a membrane. We asked if Frondoside A could inhibit PGE₂-stimulated migration. Tumor cells were placed in the upper chamber of modified Boyden chambers; FBS (positive control), PGE₂ or PGE₁-OH were present in the bottom chamber and some wells contained Frondoside A. Figure 5 confirms that PGE₂ induces the directed chemotaxis of tumor cells comparable to the positive control. Likewise, the EP4 agonist PGE₁-OH induces tumor cell migration consistent with a migration and metastasis-promoting role for EP4. Migration induced by either PGE₂ or PGE₁-OH was completely inhibited by either concentration of Frondoside A employed. Frondoside A alone had no effect on basal levels of tumor cell migration in the absence of EP ligand.

Discussion

Sea cucumbers contain many bioactive components and are widely used in traditional Chinese medicine [1]. The glycosylated triterpenoid saponins isolated from these organisms have multiple activities including anticancer activity in several models [2-5]. Frondoside A, derived from the Atlantic sea cucumber *Cucumaria frondosa* inhibits the proliferation and induces apoptosis in pancreatic cancer cells [2,3]. Frondanol-A5P, a parent compound containing several *Cucumaria frondosa*-derived glycosides, induced G2/M phase cell cycle arrest accompanied by phosphorylation of stress-activated protein kinase, Janus kinase, and p38 MAP kinase. Chemopreventive activity of Frondanol A5P was also demonstrated in an azoxy-methane-induced rat colon carcinogenesis model [4].

Based on these encouraging findings in primary tumor models, we investigated therapeutic efficacy of Frondoside A in a model of metastatic breast cancer. Distant metastatic dissemination is the overwhelming cause of death in breast cancer and there are few therapeutic strategies that specifically prevent metastasis. Very recently, migration of MDA-MB-231 cells was shown to be inhibited in a wound healing assay [5]. We now report that Frondoside A potentially inhibits metastasis *in vivo* from a mammary gland-implanted tumor. In the lung colony-forming assay, tumor cells were exposed to Frondoside A for 15-30 minutes, washed and injected i.v. into mice. Under this protocol, lung tumor colony-forming capacity was reduced indicating that a rapidly-induced mechanism contributes to the reduced metastatic potential. In the more clinically relevant model, in which tumor cells are implanted into the mammary gland and allowed to metastasize spontaneously, a comparable therapeutic response was observed. We confirmed reports in other cancers, that Frondoside A can inhibit the growth of malignant cells *in vitro* at low μM concentrations [2-5]. The related compound Frondanol A5P induces apoptosis in HCT116 cells and mediates cell cycle arrest at S and G2-M phases of the cell cycle [4]. Inhibition of pancreatic cancer cell lines is also associated with G2-M cell cycle arrest and activation of SAPK/JAK and p38 MAP kinases [3]. In spite of the demonstrated antiproliferative effect of Frondoside A, the growth of syngeneic mammary-gland implanted tumors in mice was not inhibited. Growth inhibition of MDA-MB-231 xenografts was achieved in mice treated with 100 $\mu\text{g/kg}$ Frondoside A [5] and it is possible that the doses used in the current study (10, 50 $\mu\text{g/kg}$) were suboptimal to impact tumor expansion.

We have also observed that migration of mammary tumor cells *in vitro* in response to PGE_2 or the EP4-specific ligand $\text{PGE}_1\text{-OH}$ is inhibited by Frondoside A. The inhibitory effects on both cell growth as well as migration are likely to contribute to the observed reduction in metastasis seen *in vivo*. Based on the current studies, we also propose one novel mechanism by which Frondoside A can affect tumor growth. We now show that Frondoside A is a functional antagonist of the prostaglandin E receptors, EP2 and EP4. Using cell lines engineered to express either EP2 or EP4, we found that Frondoside A inhibits binding of $^3\text{H-PGE}_2$ to either EP4 or EP2-expressing cells; EP4 binding is inhibited by Frondoside A at an estimated IC_{50} of 3.7 μM and the IC_{50} for EP2 was 16.5 μM . Consistent with these data, Frondoside A more effectively inhibited EP4-coupled versus EP2-coupled activation of adenylyl cyclase. EP4-specific ERK activation was also inhibited by Frondoside A. EP4 $-/-$ cells were less sensitive to the antiproliferative effects of Frondoside A than two cell lines expressing normal levels of EP4. Frondoside A also inhibited tumor cell migration in response to an EP4-specific ligand or to PGE_2 which can induce migration through either the EP2 or EP4 receptors (both are antagonized by Frondoside A). Taken together, these data show that Frondoside A is an effective antagonist of EP4 and, to a lesser degree, antagonizes EP2 actions. We have shown previously that either pharmacologic antagonism of the EP4 receptor or gene silencing of EP4 leads to reduced migration *in vitro* and reduced

metastatic capacity *in vivo* [8,11] and the current study supports the hypothesis that Frondoside A limits metastatic disease in part by antagonizing the EP4 receptor.

Given the concerns regarding cardiotoxicity of COX-2 inhibitors and to improve efficacy, recent studies have sought alternative therapeutic targets within the COX-2 pathway. It should be possible to avoid some of the toxicities associated with inhibition of the entire COX-2 synthetic pathway, which reduces PGE₂, but also inhibits potentially protective eicosanoids including prostacyclin. We have proposed that selective inhibition downstream of PGE₂ at the level of ligand *action* (on EP receptors) may improve the efficacy and safety profile.

There is a growing body of evidence that EP4, which is expressed on a number of different malignancies, can promote tumor metastasis. It is well established that EP receptors can promote the migration of tumor cells *in vitro* [15,16]. Using the same model of metastatic breast cancer employed in the current study, our laboratory first showed that pharmacologic antagonism or gene silencing of EP4 inhibits metastasis [11]. EP4 also promotes the invasive behavior of inflammatory breast cancers, one of the more aggressive forms of breast cancer [17]. Likewise, EP4 supports metastasis in murine models of lung and colon cancer [18]. The high expression of COX-2 in many of these tumor models could activate EP4 in an autocrine fashion [12,13]. Direct inhibition of migration is a likely mechanism underlying the antimetastatic effects of EP4 blockade. EP4 also transactivates EGFR to stimulate colon cancer metastasis [16]. Inhibition of EP4 reduces invasive properties which are linked to suppression of metalloproteinases [19]. EP4 also protects tumor cells from chemotherapy-induced apoptosis.

Many host cells also express EP receptors [20,21]. We have shown that PGE₂ suppresses the antimetastatic activity of Natural Killer cells by acting on EP4 receptors expressed on the NK cell [8, 22] and some studies show that endothelial cell migration is mediated by EP4 [23]. Thus, EP4 receptor signaling contributes to many possible mechanisms that promote tumor growth and metastasis and is therefore a potentially important therapeutic target. EP4 antagonism could prove beneficial by many mechanisms including (i) blocking tumor-expressed EP4 that contributes to growth and dissemination; (ii) angiogenesis could be suppressed by actions on EP4-mediated endothelial cell migration; (iii) Natural Killer and other immune effector cells could be protected from PGE₂-mediated immune suppression acting through EP4. In spite of the promising preclinical data, there is a paucity of selective EP4 antagonists that are available to evaluate in preclinical or clinical studies. Frondoside A may represent a novel compound that, by antagonizing EP4 and by other mechanisms, can prove useful in the metastatic setting. These studies provide a further rationale for the continued evaluation of this compound for cancer therapy.

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Frondoside A

Figure 1.
Chemical structure of Frondoside A

Figure 2.

(A) Line 66.1 tumor cells treated with Frondoside A (FA, 5.0 $\mu\text{M/L}$) or vehicle for 15 minutes, washed and 1×10^5 viable cells injected i.v. into the lateral tail vein of Balb/cByJ female mice (10 mice /group). On day 21, mice were euthanized and surface lung tumor colonies were quantified. (B) Tumor cells treated with Frondoside A (0.1 or 1.0 $\mu\text{M/L}$) for 30 minutes, washed and injected as in A. (C) Five $\times 10^5$ line 66.1 tumor cells injected s.c. proximal to the right abdominal mammary gland and Frondoside A or vehicle administered i.p. at either 10 or 50 $\mu\text{g/kg}$ beginning on day of tumor transplantation and continuing for 10 days. Tumor growth monitored by caliper and mice euthanized on an individual basis when tumors measure 18 mm in average diameter and lung tumor colonies quantified. P-value from the nonparametric exact Wilcoxon test done at the two-sided 0.05 level of significance.

Figure 3.

Tumor cells treated with indicated agonists or antagonists for 15 min. and intracellular cAMP levels determined. **(A)** PGE₂ (5 μM/L), AH23848 (5 μM/L) or Frondoside A (FA, 0.1, 1.0 or 5.0 μM/L) added to cells. **(B)** PGE₁-OH (5 μM/L) AH23848 (5 μM/L) or Frondoside A (FA, 0.1, 1.0 or 5.0 μM/L) added to wells. **(C)** butaprost (1 μM/L), AH6809 (1 μM/L) or Frondoside A (0.1, 1.0 or 5.0 μM/L) added to cells. Comparison of means by Student's t-test. **(D)** Cells pretreated with Frondoside A or vehicle followed by treatment with PGE₂ (5 μM/L) or PGE₁-OH (5 μM/L) for 15 minutes and levels of ERK1/2 and phosphorylated ERK1/2 determined by western blotting of cell lysates and expressed as fold-stimulation relative to untreated cells.

Figure 4.

(A) Parental 66.1 cells (1.5×10^5) added to wells and on day +1, vehicle or the indicated concentrations of Frondoside A were added. Cell number was determined 24 hrs later. (B) 66.1-vector (black bar) or 66.1shEP4 cells (grey bar) plated at 1.5×10^5 cells per well and on day +1, Frondoside A or vehicle added at the indicated concentrations and cell number determined on day +2. Results plotted as percent of cell number in Frondoside A-treated versus vehicle-control treated cells. Comparison of means by Student's t-test.

Figure 5.

Calcein-labeled tumor cells placed in upper well of Boyden chamber and FBS (2%), PGE₂ (5 μ M/L), PGE₁-OH, (5 μ M/L) and Frondoside A (FA, 0.1, 1.0 μ M/L) placed in the lower chamber. Cells were allowed to migrate for 24 hours and the number of tumor cells in the lower chamber was determined by fluorescence at 485 nM. Data plotted as the fold migration in comparison to basal migration in the absence of stimulant. Comparison of means by Student's t-test.

Table 1Inhibition of [³H]-PGE₂ Binding by Frondoside A

Receptor	Binding Affinity	Percent Inhibition
Chem-1-EP4	0.69 nM Bmax=4.3 pmole/mg protein	FA 1 µg/ml=21%
		FA 10 µg/ml=94%
HEK-293-EP2	3.1 nM Bmax=1.4 pmole/mg protein	FA 20 µg/ml=48%
		FA 40 µg/ml=77%