

Agelastatin A: a novel inhibitor of osteopontin-mediated adhesion, invasion, and colony formation

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Abstract

Effective inhibitors of osteopontin (OPN)-mediated neoplastic transformation and metastasis are still lacking. (-)-Agelastatin A is a naturally occurring oroidin alkaloid with powerful antitumor effects that, in many cases, are superior to cisplatin *in vitro*. In this regard, past comparative assaying of the two agents against a range of human tumor cell lines has revealed that typically (-)-agelastatin A is 1.5 to 16 times more potent than cisplatin at inhibiting cell growth, its effects being most pronounced against human bladder, skin, colon, and breast carcinomas. In this study, we have investigated the effects of (-)-agelastatin A on OPN-mediated malignant transformation using mammary epithelial cell lines. Treatment with (-)-agelastatin A inhibited OPN protein expression and enhanced expression of the cellular OPN inhibitor, Tcf-4. (-)-Agelastatin A treatment also reduced β -catenin protein expression and reduced anchorage-independent growth, adhesion, and invasion in R37 OPN pBK-CMV and C9 cell lines. Similar effects were observed in MDA-MB-231 and MDA-MB-435s human breast cancer cell lines exposed to (-)-agelastatin A. Suppression of Tcf-4 by RNA interference (short interfering RNA) induced malignant/invasive transformation in parental benign Rama 37 cells; significantly, these events were reversed by treatment with (-)-agelastatin A. Our study reveals, for the very first time, that (-)-agelastatin A down-regulates β -catenin expression while simultaneously up-regulating Tcf-4 and

that these combined effects cause repression of OPN and inhibition of OPN-mediated malignant cell invasion, adhesion, and colony formation *in vitro*. We have also shown that (-)-agelastatin A inhibits cancer cell proliferation by causing cells to accumulate in the G₂ phase of cell cycle. [Mol Cancer Ther 2008;7(3):548–58]

Introduction

Metastasis is a complex process of genetic and phenotypic change that ultimately results in tumor cell dissemination and the formation of secondary tumors (1). The adhesive glycoprotein osteopontin (OPN) has been heavily implicated in exacerbating the processes of neoplastic transformation, cancer progression, and metastasis (2) and as such is of considerable oncologic interest.

OPN is transcriptionally regulated by a variety of Wnt signaling factors that include Tcf-4 and β -catenin (3). In normal cells, the levels of β -catenin, Tcf-4, and OPN are all very tightly regulated, whereas in many metastatic cancer cells the expression of all three proteins is significantly deregulated or aberrant, with the extent of abnormality usually correlating with final prognostic outcome. For example, in primary human breast carcinoma, patient demise is typically associated with a dramatic increase in β -catenin and OPN levels and a significant down-regulation of Tcf-4, if one makes comparisons with normal, healthy, human breast tissue (2).

Numerous studies have now established that when β -catenin is overexpressed within cells, it translocates to the nucleus where it forms an oncogenic complex with Tcf-4 to drive the processes of transcription from various genes involved in cancer cell growth and proliferation as well as metastasis, including the OPN gene (see refs. 4–8). Mutated β -catenin proteins have also been identified that behave as functional mimics of their unmutated counterparts, and these become especially problematical when they can successfully evade down-regulation and degradation by functionally effective, tumor-suppressing, APC destruction complexes (7); such mutated β -catenins are usually oncogenic (7).

Because OPN is now widely believed to be one of the key transcriptional targets of the β -catenin/Tcf-4 complex (3, 9) involved in metastasis, and activated β -catenin can induce OPN expression in migrating tumor cells (9), disruption of the β -catenin/Tcf-4 complex and the controlled down-regulation of overexpressed β -catenin within such cells is now considered to be an exciting strategy for potentially combating metastatic cancer spread, most especially if it can be accomplished with small-molecule drugs (6).

Previous work from our laboratory has shown that in benign rat mammary epithelial cells (Rama 37) low levels of OPN are accompanied by a high expression of Tcf-4 (3).

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However, in metastatic Rama 37 Met-DNA (C9) cells, high OPN levels are always observed alongside fairly low levels of Tcf-4 (3). Both of these cell lines provide excellent models for studying the processes of malignant transformation and metastasis (3).

In recent years, the quest for new anticancer drugs has led researchers to search various marine ecosystems for structurally unique tumor-inhibitory compounds (10). One recently discovered anticancer agent of marine origin is the unusual brominated oroidin alkaloid, (-)-agelastatin A, obtained from extracts of the axinellid sponge *Agelas dendromorpha* (11–13). It potently inhibits the growth of murine and human cancer cell lines at low drug concentrations (11–15) by mechanisms that have yet to be fully delineated. In this regard, (-)-agelastatin A potently retards the growth of a range of human tumor cell lines that include KB nasopharyngeal cancer cells (IC₅₀, 0.075 µg/mL), RT112/84 bladder carcinoma cells, SK-MEL-5 melanoma cells, HCT-116 colon carcinoma cells, and MDA-MB-435s breast cancer cells (15). In the latter four cell lines, (-)-agelastatin A is between 3 and 16 times more potent as an anticancer drug than the frontline chemotherapeutic agent cisplatin (15). Preliminary toxicologic evaluations of (-)-agelastatin A, in tumor-implanted mice, have shown that the molecule can be given safely when it is administered at doses of 2.5 mg/kg/d (15). Other studies have also confirmed that when (-)-agelastatin A is administered at 2.6 mg/kg/d, it can typically confer a 65% increase in lifespan on mice with murine L1210 leukemia (13).

As part of a general effort to elucidate the mechanism of antitumor action of (-)-agelastatin A, we recently began investigating its effects on the expression of β-catenin, Tcf-4, and OPN within a range of cells. We also examined its effect *in vitro* on OPN-mediated malignant transformation in various mammary epithelial cell models. OPN-independent effects of (-)-agelastatin A on cell growth and cell cycle progression were further assessed as well. Herein, we now report the results of these combined studies, which provide fascinating new insights into the unique mode of antitumor action of this novel natural product.

Materials and Methods

(-)-Agelastatin A

(-)-Agelastatin A was chemically synthesized as described previously by our group (15, 16). It was dissolved in DMSO at a concentration of 1 mmol/L and stored at -20°C. Aliquots of this solution were subsequently diluted and made up to the appropriate concentration before treatment of cells.

Cell Lines and Cell Culture

The Rama 37 and the C9 Met-DNA permanently transfected Rama 37 cells (C9) were obtained and cultured as described previously (17, 18). The pBK-CMV vector containing OPN was permanently transfected into Rama 37 cells in this laboratory (R37 OPN pBK-CMV). Breast cancer cells MDA-MB-231 and MDA-MBA-435s were obtained from the European Collection of Cell Cultures.

All cell lines were maintained in a humidified atmosphere of 95% (v/v) air and 5% (v/v) CO₂ at 37°C in routine medium (DMEM; Sigma) containing 10% (v/v) FCS, 100 µg/mL penicillin, and 100 µg/mL streptomycin (Life Technologies).

Treatment of Cells with (-)-Agelastatin A

Before treatment with (-)-agelastatin A, cells were grown overnight in routine medium. The next day, (-)-agelastatin A in DMSO was added to a final concentration of 10 nmol/L in routine medium unless otherwise specified. Cells were treated for 48 or 72 h before assaying.

Cell Growth Assays

Cell growth assays were carried out by plating out 1×10^5 cells per well of a six-well plate. At time points of 3, 6, 12, 24, 48, 72, and 96 h, cells were removed by trypsination and counted using a hemocytometer. All assays were carried out in triplicate as described previously (19).

Western Blotting for Proteins

For the detection of proteins, 15 µg whole-cell lysates were electrophoresed through 10% (w/v) polyacrylamide, 1% (w/v) SDS gels. Proteins were transferred from the gels by blotting onto a nitrocellulose membrane (Millipore). The membranes were blocked with 0.02 mol/L Tris-HCl (pH 7.0), 0.9% (w/v) NaCl, 0.1% (v/v) Tween 20 containing 5% (w/v) Marvel for 1 h. Monoclonal antibodies to OPN (1:500; Developmental Studies Hybridoma Bank), Tcf-4 (1:500; Upstate), β-catenin, cyclin B1 (1:1,000; Santa Cruz Biotechnology), cyclin D1 (1:500), cyclin E (1:200; Calbiochem), or β-actin (1:5,000; Sigma) were added and incubated overnight at 4°C. Bound antibodies were located by a further incubation with 1:2,500 horseradish peroxidase-conjugated rabbit anti-mouse IgG (for anti-OPN/Tcf-4/β-actin; Dako) or 1:2,500 horseradish peroxidase-conjugated donkey anti-mouse IgG (for anti-β-catenin; Santa Cruz Biotechnology), visualized with Western Blotting Luminol Reagent (Santa Cruz Biotechnology), and exposed to Kodak XAR5 film (Sigma). Densitometry data were obtained using a digital imaging system (Syngene, Genetool). All Western blot experiments were repeated at least three times to ensure reproducibility as described previously (19, 20).

Cell Adhesion Assay

The adhesive ability of cells was determined as described previously (19, 21). Briefly, cells were plated out at a known density (2×10^5 per dish) in a 24-well plate in conditioned medium and allowed to adhere to laminin-coated plates for 15 min at 37°C, 5% CO₂. Cells were washed with PBS, fixed with methanol, and stained with crystal violet (Sigma). The stain was released by 10% (v/v) acetic acid and cell adhesion was determined by measuring the absorbance at 570 nm and compared with controls. Three independent experiments were carried out in triplicate to ensure reproducibility.

Soft Agar Assays

Anchorage-independent growth was determined as described previously (19). Briefly, 5 mL of 1.6% (w/v) agarose were plated in a 100-mm diameter tissue culture dish and allowed to harden. Cells were removed by

trypsinization and resuspended at 1×10^6 cells/mL in routine medium. To the agar 9 mL routine medium [containing (-)-agelastatin A for treated samples] and 1 mL cells were added. The plates were incubated at 37°C in 5% (v/v) CO₂ for 5 to 7 days and stained with 1 mL of 0.2% (w/v) crystal violet. The plates were scanned for colonies and counted using a digital imaging system (Syngene).

Matrigel Invasion Assays

Biocoat 250 µg/mL Matrigel invasion chambers (diameter, 6.4 mm; Falcon) were used to assess the invasiveness of cells as described previously (19). Briefly, 1×10^6 cells were resuspended in 100 µL serum-free DMEM [containing (-)-agelastatin A for treated samples] and added to the cell culture inserts of the upper invasion chambers. A chemo-attractant, 5 µg rat fibronectin (Life Technologies)/mL in DMEM and 10% (v/v) FCS were added to the lower chambers. The cultures were incubated at 37°C in 5% (v/v) CO₂ atmosphere and allowed to invade through the matrix and the pores (8 µm) of the attached lower membrane for 48 h. Following incubation, the upper surfaces of the filters were wiped clean of cells and the filters were fixed with methanol and stained by Gurr's eosin and methylene blue according to the manufacturer's instructions (BDH Laboratory Supplies). The chambers were then treated with 10% (v/v) acetic acid to release the stain and the absorbance was measured at 650 nm using a microtiter plate reader (Molecular Devices).

OPN Promoter Studies and Plasmids

Expression vectors for human Tcf-4 and Lef-1 in the pcDNA3 were gifts from Prof. H. Clevers (University of Utrecht). The expression vector for human β-catenin in pcDNA3 was a gift from Prof. B. Vogelstein and Dr. K. Kinzler (John Hopkins University). Rat genomic DNA was donated by Dr. A. Ridall (Department of Basic Sciences, University of Texas Houston-Health Science Center) and was used as template for isolation of the OPN promoter as described previously (22).

Transient Transfections

Rama 37 cells cultured in routine medium were harvested and seeded in 24-well plates at 2×10^5 per well in 1 mL serum-free medium. After 24 h, the cells were cotransfected using LipofectAMINE and PLUS Reagent (Invitrogen) with predetermined amounts of the following, where indicated: 25 ng Tcf-4 expression vector, 12.5 ng β-catenin and Lef-1 expression vectors, and 75 ng OPN promoter. The control expression vector pRL *Renilla* (Promega) at 2.5 ng was used as a control expression vector. The cells were incubated for a further 48 h [with (-)-agelastatin A for treated samples] and harvested in 300 µL Reporter Lysis Buffer (Promega), and firefly luciferase and control *Renilla* luciferase were simultaneously assayed as described in the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Data were analyzed by calculating mean fold activation of 10 nmol/L (-)-agelastatin A-treated cells compared with the average of untreated control as described previously (22).

RNA Interference (Short Interfering RNA)

RNA interference was carried out using the Super RNAi library (Cancer Research UK; refs. 19, 23). Briefly, the 19-mer sequences from the Tcf-4 gene were converted into pairs of complementary 59-mer hairpin oligonucleotides. The complementary 59-mer oligonucleotides targeting the Tcf-4 gene were annealed and ligated into the pRETROSUPER vector and transfected into competent DH5 bacteria. Glycerol stocks of transformants were prepared with the well containing bacteria with the short interfering RNA (siRNA) construct targeting the Tcf-4. DNA from the siRNA construct was isolated using the Qiagen mini-prep system and confirmed by DNA sequencing. The three siRNA oligonucleotide sequences for Tcf-4, designed according to the human mRNA sequence (GenBank accession no. BC125085), were as follows: 5'-GCCCGTCCAGGAAGTATGG-3', 5' CCATTACAGCACC-TCTTC-3', and 5'-GGAGGCCTTTCACAGTAG-3'. This DNA was subsequently used for stable transfection into Rama 37 cells (with LipofectAMINE and PLUS Reagent), with selection by puromycin (Sigma; ref. 19).

Cell Cycle Distribution Analysis

The cell cycle distribution was determined by propidium iodide staining and analysis by flow cytometry as described previously (24). Briefly, following 72-h post-treatment with (-)-agelastatin A, floating and adherent cells were collected, washed twice with PBS/1% serum, and fixed with ice-cold absolute ethanol overnight at 4°C. Cells were washed in PBS/1% serum and resuspended in a propidium iodide solution (10 µg/mL) containing RNase A (25 µg/mL). Cells were stained for 30 min at 37°C in the dark and then analyzed using a BD FACS Canto II flow cytometer (Becton-Dickinson). Cell cycle distribution was assessed on DNA plots using FlowJo software.

Statistical Treatment of Results

All biological experiments were done at least three times. The mean and SE were calculated and *P* values ≤ 0.05 were considered significant as calculated using the Student's *t* test.

Results

Effect of (-)-Agelastatin A on Protein Expression

The effect of (-)-agelastatin A on the expression of OPN and various Wnt signaling proteins (Fig. 1A and B) was assessed by Western blotting. (-)-Agelastatin A treatment suppressed OPN and β-catenin expression but promoted Tcf-4 protein expression. Treatment of cells with 10 nmol/L (-)-agelastatin A suppressed OPN expression by 10% (data not shown), whereas the reduction of OPN was maximal (93% reduction in R37 OPN pBK-CMV and 60% in C9 cells) when cells were treated with 1 µmol/L (-)-agelastatin A (Fig. 1A). A notable decrease in β-catenin expression was also observed (R37 OPN pBK-CMV, 98%; C9 cells, 50%). A 3-fold increase was noted in Tcf-4 protein expression in R37 OPN pBK-CMV cells and a 1.5-fold increase was detected in C9 cells at concentrations as low as 10 nmol/L (Fig. 1A).

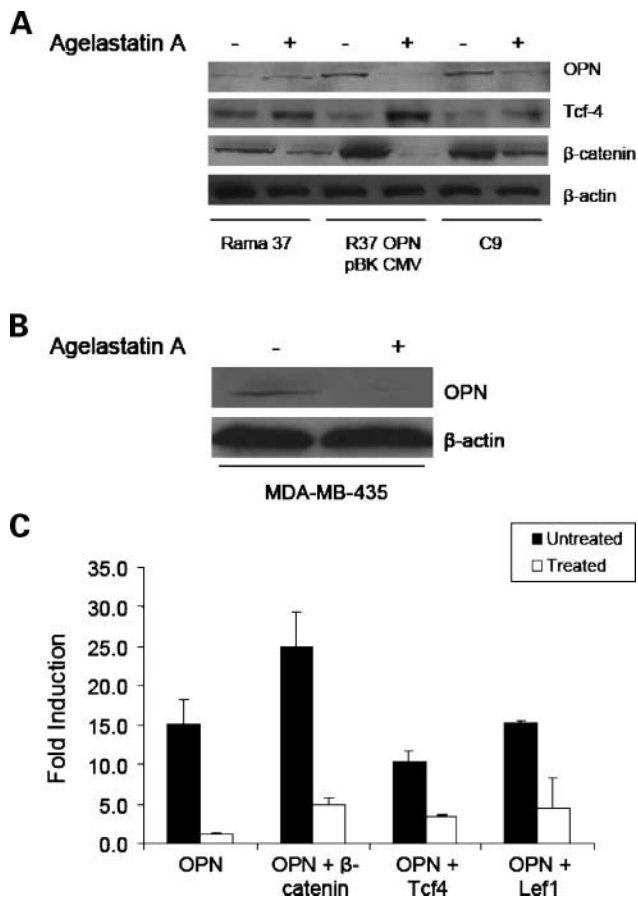


Figure 1. **A**, Western blots showing OPN, Tcf-4, and β -catenin protein levels in R37, R37 OPN pBK-CMV, and C9 cells. Cell lysates were diluted and 15 μ g were loaded onto a SDS 10% (w/w) polyacrylamide gel. Specific proteins were detected using antibodies to OPN, Tcf-4, β -catenin, and β -actin. Bands were quantified using densitometric analysis and normalized against β -actin. Densitometric analysis showed that treatment with (-)-agelastatin A resulted in the following: OPN protein expression was reduced by 93% in R37 OPN pBK-CMV and 60% in C9 cells. β -catenin expression was reduced by 98% in R37 OPN pBK-CMV and 50% in C9 cells. Tcf-4 protein expression was increased 3-fold in R37 OPN pBK-CMV and 1.5-fold in C9 cells. **B**, Western blots showing OPN protein levels in MDA-MB-435s cells. Cell lysates were diluted and 15 μ g were loaded onto a SDS 10% (w/w) polyacrylamide gel. Specific proteins were detected using antibodies to OPN and β -actin. Bands were quantified using densitometric analysis and normalized against β -actin. Densitometric analysis showed that treatment with (-)-agelastatin A in MDA-MB-435s cells resulted in a 83% reduction in OPN protein expression. **C**, effect of (-)-agelastatin A on OPN promoter-linked luciferase activity. Rama 37 cells were cotransfected with the OPN promoter luciferase reporter and with various Wnt signaling proteins in expression vectors (β -catenin, Tcf-4, and Lef-1). Mean \pm SD of fold induction of luciferase activity of three independent experiments.

Treatment of MDA-MB-435s cells with 1 μ mol/L (-)-agelastatin A reduced OPN protein expression by 83% as determined by densitometry (Fig. 1B).

Effect of (-)-Agelastatin A on Transcriptional Regulators of the OPN Promoter

To further assess the effects of (-)-agelastatin A on transcriptional regulators acting at the level of the OPN promoter, cotransfection experiments were done (Fig. 1C).

In untreated Rama 37 cells, cotransfection of an OPN promoter luciferase construct (OPN-luc) with β -catenin in an expression vector significantly enhanced the luciferase activity of the OPN reporter construct by an additional 1.6-fold. Cotransfection of OPN-luc with Tcf-4 in an expression vector resulted in a decrease in OPN promoter activity by 58% compared with transfection of OPN-luc with β -catenin. Treatment of cells cotransfected by OPN-luc and the various transcriptional regulators with (-)-agelastatin A further reduced luciferase activity. Treatment with (-)-agelastatin A significantly reduced the luciferase activity of the OPN promoter by 92%, β -catenin by 80%, Tcf-4 by 67%, and Lef-1 by 70% (Fig. 1C).

Effect of (-)-Agelastatin A on Growth of Rama 37, C9, MDA-MB-231, and MDA-MB-435s Mammary Cells

Growth of benign rat mammary (Rama 37) cells and the metastatic C9 subclone was assessed with varying concentrations of (-)-agelastatin A (Fig. 2A and B). When treated with (-)-agelastatin A at concentrations of 10 and 100 nmol/L, the growth of both Rama 37 and C9 cells was similar to that of untreated cells. However, at a concentration of 1 μ mol/L, there was virtually no growth of either Rama 37 or C9 cells. At a midpoint concentration of 500 nmol/L, the growth of Rama 37 (Fig. 2A) and C9 (Fig. 2B) cells slowed by \sim 60% in both cell types compared with untreated cells or those exposed to 10 nmol/L concentrations of the drug (Fig. 2A and B). (-)-Agelastatin A treatment inhibited the growth of MDA-MB-231 and MDA-MB-435s cells in a dose-dependent manner in both cell lines (Fig. 2C and D).

Effect of (-)-Agelastatin A on Cell Adhesion, Colony Formation, Migration, and Invasion

Various biological assays have been developed to assess the metastatic potential of cells *in vitro*. In this current study, all of the biological assays employed revealed that (-)-agelastatin A could significantly reduce the metastatic potential of cells (Fig. 3).

A key feature of OPN-mediated metastasis is the adhesion of tumor cells to extracellular matrix components (25). Rama 37 cells have been proven previously to adhere at low levels, and in this study, although (-)-agelastatin A did reduce adhesion, it did not do so significantly ($P = 0.217$). However, in OPN pBK-CMV cells, a reduction of 46% was observed on treatment with (-)-agelastatin A at 10 nmol/L ($P = 0.041$). In the case of the metastatic C9 cells (the most adherent cell line), treatment with (-)-agelastatin A at 10 nmol/L significantly reduced adhesion of the cells by 47% ($P = 0.003$). No further decrease in adhesion was noted on treatment with 0.5 or 1 μ mol/L concentrations of (-)-agelastatin A (Fig. 3A).

The ability of tumor cells to grow in soft agar correlates with the tumorigenic potential of cells through anchorage-independent growth (26). Although OPN has been proven to increase the ability of cells to grow in soft agar (19, 27), the mechanism by which it does this has yet to be elucidated (27). In this study, as expected, Rama 37 cells showed low levels of anchorage-independent growth, and (-)-agelastatin A had no significant effect on colony

formation ($P = 0.074$). On the other hand, in both R37 OPN pBK-CMV and C9 cells, which contain high levels of OPN, a high rate of proliferation was observed in soft agar assays. Moreover, in R37 OPN pBK-CMV and C9 cells, treatment with (-)-agelastatin A at 10 nmol/L concentration significantly reduced colony formation in soft agar by 81% and 48%, respectively ($P = 0.048$ and 0.016 ; Fig. 3B).

The benign Rama 37 cell line has been shown previously to be nonmetastatic and unable to invade or migrate to distant sites (17). Treatment of this cell line with (-)-agelastatin A showed no significant effect on the migratory or invasive potential of these cells ($P = 0.127$ and 0.260). In Rama 37 cells, permanently transfected with OPN and proven to be invasive *in vitro* (19), (-)-agelastatin A (10 nmol/L) significantly reduced the migration and invasive ability of these cells by 51% and 40%, respectively ($P = 0.039$ and 0.028 , respectively). In C9 cells, which are metastatic in both *in vitro* and *in vivo* animal setting (21), treatment with (-)-agelastatin A at 10 nmol/L concentration very significantly reduced their migratory and invasive potential by 49% and 61%, respectively ($P = 0.011$ and $8.47E-04$; Fig. 3C and D).

In vitro biological assays were also carried out to investigate the effect of (-)-agelastatin A on the metastatic potential of human breast cancer cells as judged by

adhesion, invasion, and colony formation (Fig. 3). Treatment of MDA-MB-231 with 10 nmol/L (-)-agelastatin A significantly reduced adhesion by 43% ($P = 0.0014$). Likewise, treatment of MDA-MB-435s cells with (-)-agelastatin A markedly reduced adhesion in a concentration-dependent manner with a maximal reduction of 56% achieved after treatment with 1 μ mol/L (-)-agelastatin A ($P = 2.64E-05$; Fig. 3E). (-)-Agelastatin A treatment also inhibited anchorage-independent growth in MDA-MB-231 and MDA-MB-435s cells by 37% and 25% ($P = 0.085$ and 0.050 , respectively; Fig. 3F) and reduced invasion of MDA-MB-435s by 36% ($P = 0.034$ and 0.015 ; Fig. 3G).

RNA Interference

To discern the specific role of Tcf-4 and OPN transcription as well as OPN protein expression and OPN-mediated neoplastic transformation following treatment with (-)-agelastatin A, RNA interference strategies were employed (19, 28, 29). Stable transfection of Rama 37 cells with a Tcf-4 pSUPER RNAi expression vector impeded Tcf-4 protein expression and was associated with increased OPN protein levels (Fig. 4A).

Interestingly, treatment of Tcf-4 siRNA stable transfectants with (-)-agelastatin A partially reversed the effects of Tcf-4 siRNA and induced a 3-fold increase in Tcf-4 protein expression accompanied by a 63% decrease in OPN protein

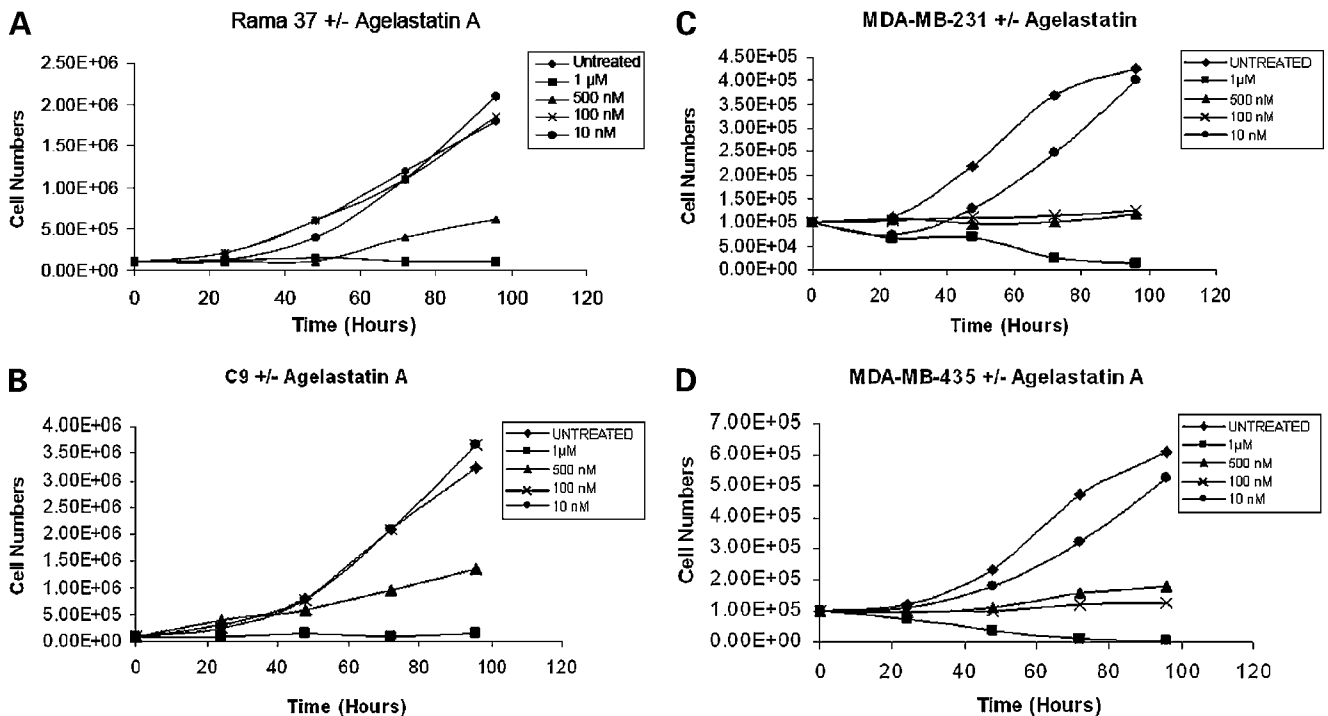


Figure 2. **A**, effect of varying concentrations of (-)-agelastatin A on cell proliferation in Rama 37 cells. 1×10^5 cells were seeded in six-well plates and cell numbers were counted at regular time intervals. Mean of three independent experiments. **B**, effect of varying concentrations of (-)-agelastatin A on cell proliferation in C9 cells. 1×10^5 cells were seeded in six-well plates and cell numbers were counted at regular time intervals. Mean of three independent experiments. **C**, effect of varying concentrations of (-)-agelastatin A on cell proliferation in MDA-MB-231 cells. 1×10^5 cells were seeded in six-well plates and cell numbers were counted at regular time intervals. Mean of three independent experiments. **D**, effect of varying concentrations of (-)-agelastatin A on cell proliferation in MDA-MB-435s cells. 1×10^5 cells were seeded in six-well plates and cell numbers were counted at regular time intervals. Mean of three independent experiments.

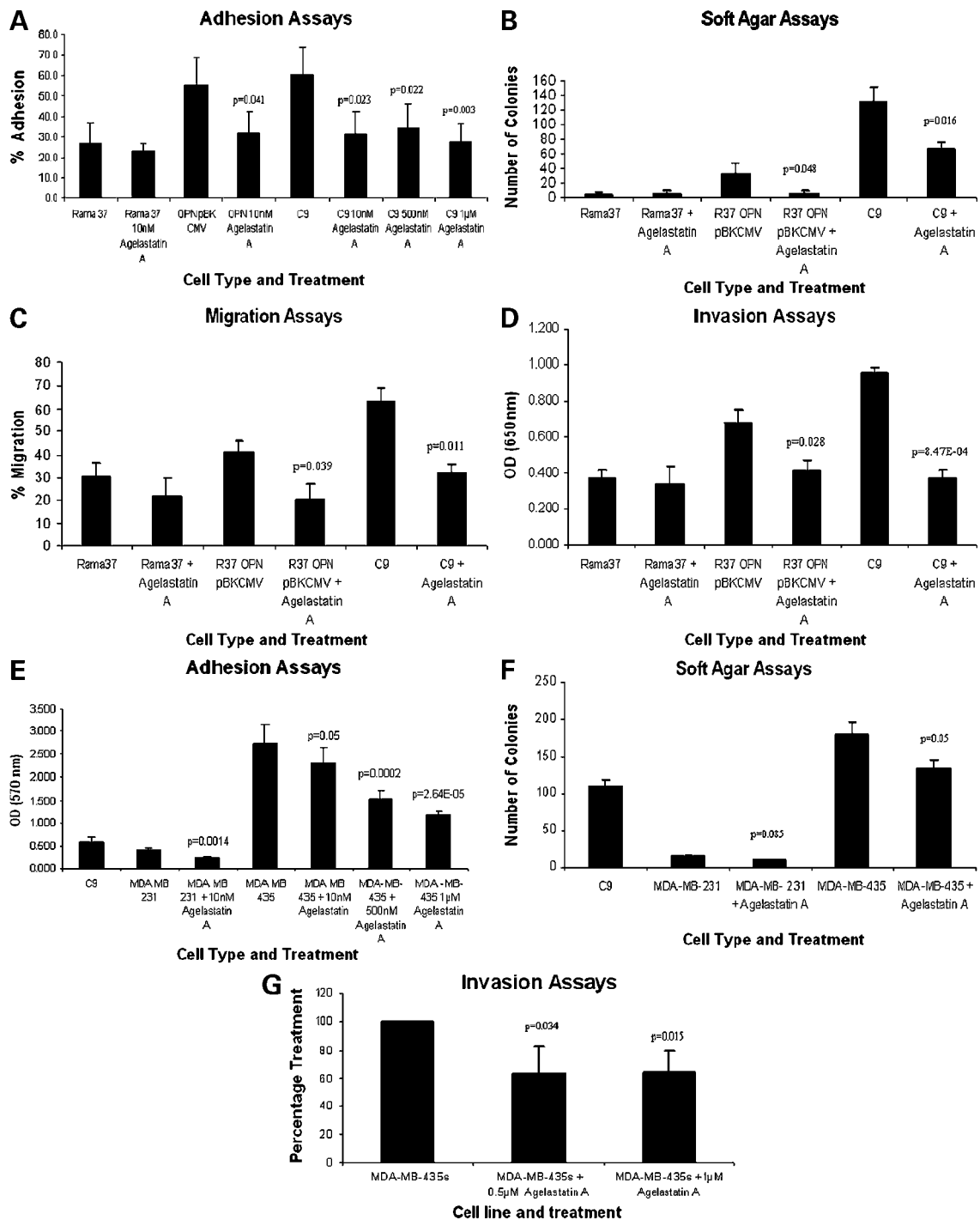


Figure 3. **A**, ability of cell lines to adhere to a laminin-treated surface was assessed over a 30-min period and the number of adherent cells was quantified. Mean \pm SD of three independent experiments. **B**, a soft agar assay was carried out to assess the ability of stably transfected cell lines to grow in an anchorage-independent environment. The colony number was assessed after 5 d. Mean \pm SE of three independent experiments. **C**, migratory potential of R37, R37 pBK-CMV, and R37 OPN was determined using Boyden chambers without Matrigel. The number of cells that migrated through the filter after 48 h was determined by staining and scanning using a digital imaging system. Mean \pm SD of three independent experiments. **D**, invasive potential of R37, R37 pBK-CMV, and R37 OPN was determined using Matrigel-coated filters (500 μ g/mL) in Boyden chambers. The number of cells that invaded through the filter after 48 h was determined by staining and scanning using a digital imaging system. Mean \pm SD of three independent experiments. **E**, ability of cell lines to adhere to a laminin-treated surface was assessed over a 30-min period and the number of adherent cells was quantified. Mean \pm SD of three independent experiments. **F**, a soft agar assay was carried out to assess the ability of cell lines to grow in an anchorage-independent environment. The colony number was assessed after 5 d. Mean \pm SD of three independent experiments. **G**, invasive potential of MDA-MB-435s cells through Matrigel was determined using modified Boyden chambers. Mean \pm SD as a percentage of control from three independent experiments.

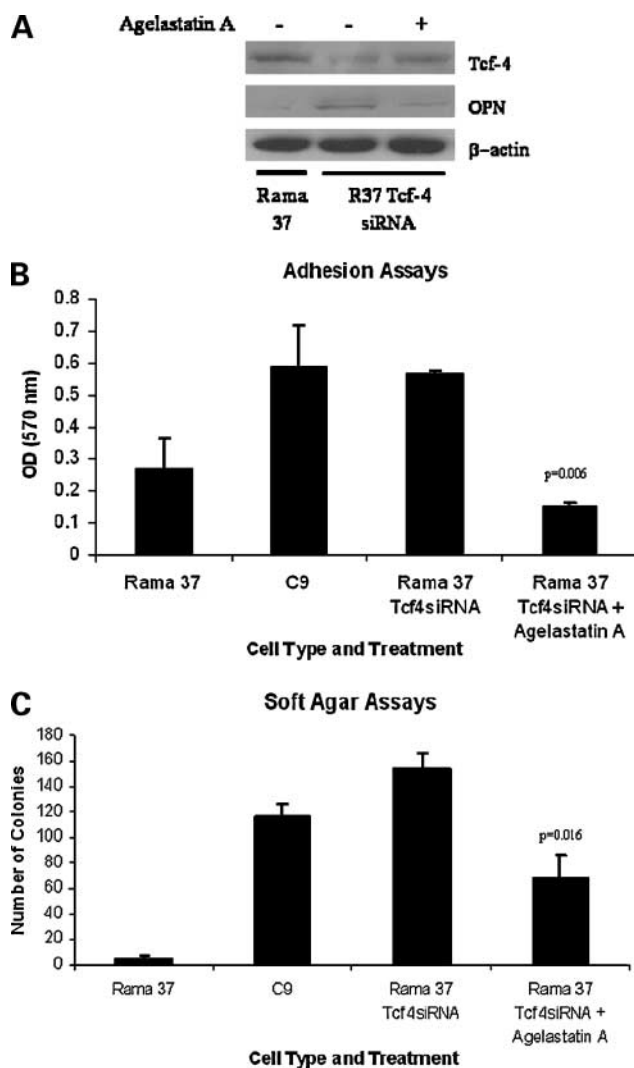


Figure 4. **A**, Western blots showing OPN protein levels in R37 and R37 Tcf-4 siRNA cells. Cell lysates were diluted and 15 μ g were loaded onto a SDS 10% (w/w) polyacrylamide gel. Specific proteins were detected using antibodies to OPN and β -actin. Bands were quantified using densitometric analysis and normalized against β -actin. **B**, ability of transfected cell lines to adhere to a laminin-treated surface was assessed over a 30-min period and the number of adherent cells was quantified. Mean \pm SE of three independent experiments. **C**, a soft agar assay was carried out to assess the ability of stably transfected cell lines to grow in an anchorage independent environment. The colony number was assessed after 5 d. Mean \pm SE of three independent experiments.

expression (Fig. 4A). Rama 37 cells stably transfected with Tcf-4 siRNA showed increased adhesion and anchorage-independent growth over that of parental Rama 37 cells (Fig. 4B and C). Treatment of Rama 37-Tcf-4 siRNA stable transfectants with (-)-agelastatin A at 10 nmol/L reduced adhesion by 74% ($P = 0.006$). In soft agar assays, this trend was repeated. The Tcf-4 siRNA Rama 37 cells showed an increase in anchorage-independent growth by 31-fold ($P = 0.016$) compared with Rama 37 cells, which was comparable with C9 cells. Treatment with (-)-agelastatin A significantly reduced this effect by a further 55% ($P = 0.016$).

Effects of (-)-Agelastatin A on Cell Cycle Progression

The effect of (-)-agelastatin A on progression of MDA-MB-435s cells through the cell cycle was determined by propidium iodide staining and analysis by flow cytometry (24) following 72-h exposure to (-)-agelastatin A. Treatment with (-)-agelastatin A induced an accumulation of cells in the G_2 phase of the cell cycle, increasing the proportion of cells in G_2 from 18% in untreated cultures to a maximum of 40% in cells treated with 1 μ mol/L (-)-agelastatin A (Fig. 5A). The increase in the proportion of cells in G_2 was sustained up to 96-h posttreatment with (-)-agelastatin A. There was a corresponding decline in the population of cells in G_1 phase from 54% in untreated cells to 31% in cells treated with 1 μ mol/L (-)-agelastatin A. There was also some evidence of increased cell death accompanying the G_2 -M arrest as indicated by the appearance of a sub- G_1 peak in the cell cycle profiles, with a 7% increase in the percentage sub- G_1 cells.

Because (-)-agelastatin A induced alterations in cell progression through the G_2 -M phase, Western blotting was used to assess the effect of (-)-agelastatin A on the expression of cyclin B1, which regulates transition through the G_2 checkpoint. It was found that (-)-agelastatin A treatment correlated with a significant concentration dependent increase in the expression of cyclin B1 protein in MDA-MB-435s cells, consistent with the known increase in cyclin B1 levels during the G_2 phase. In accordance with the subsequent decline in the number of cells in G_1 phase, we also detected a significant decrease in expression of cyclin D1 and E following treatment with (-)-agelastatin A (Fig. 5B). We have observed a similar effect with (-)-agelastatin A on cyclin D1 and B1 expression in C9 cells (Fig. 5C).

Discussion

It is now well established that high levels of OPN are associated with poor patient survival rates in breast cancer (2, 30). One of the controllers of OPN expression within cells is the Wnt signaling pathway, which has, at its heart, two key components: β -catenin and Tcf-4 (3). Typically, high levels of β -catenin and low levels of Tcf-4 are associated with the tumorigenic phenotype (3).

The ability of (-)-agelastatin A to reduce OPN and β -catenin levels within cancer cells while simultaneously increasing Tcf-4 expression is a novel property of this molecule that could be of potential future clinical relevance to the treatment of metastatic human cancer. It has been shown previously that increased levels of β -catenin protein are associated with a decrease in Tcf-4 protein (31), a phenomenon that was ascribed to different locations of β -catenin within cells. This is the first time, however, that such an association has been confirmed through modulation of these two proteins with a small-molecule anticancer drug. The down-regulation of β -catenin that we observed in the present study, with (-)-agelastatin A, might simply be due to its ability to promote enhanced degradation of the protein. However, it might also be due

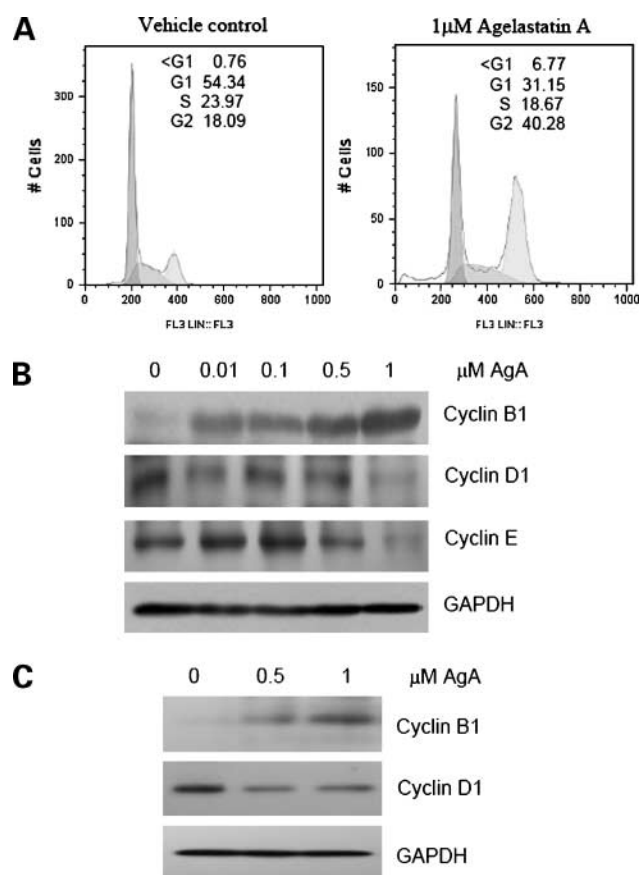


Figure 5. Effect of (-)-agelastatin A on progression of MDA-MB-435s cells through the cell cycle and expression of cell cycle regulatory proteins. **A**, flow cytometry profile showing accumulation of MDA-MB-435s cells in G₂ phase of the cell cycle following exposure to 1 μmol/L (-)-agelastatin A for 72 h. **B**, Western blot showing concentration-dependent increase in cyclin B1 expression and corresponding decrease in cyclin D1 and E following exposure of the cells to (-)-agelastatin A for 72 h. Densitometric analysis showed that treatment with (-)-agelastatin A resulted in the following: cyclin B1 protein expression was increased in a concentration-dependent manner by 29%, 38%, 61%, and 107% following treatment with 0.01, 0.1, 0.5, and 1 μmol/L (-)-agelastatin A, respectively. Cyclin D1 and E protein expression was decreased by a maximum of 37% and 78%, respectively, following treatment with 1 μmol/L (-)-agelastatin A. **C**, Western blot showing similar effect of (-)-agelastatin A on expression of cyclin B1 and D1 in C9 cells. Densitometric analysis showed that treatment with (-)-agelastatin A resulted in the following: cyclin B1 protein expression was increased in a concentration-dependent manner by 5% and 13% following treatment with 0.5 and 1 μmol/L (-)-agelastatin A, respectively. Cyclin D1 protein expression was decreased by 66% and 50% following treatment with 0.5 and 1 μmol/L (-)-agelastatin A, respectively.

to the drug causing reduced levels of the protein to be translated. Further work will be required to distinguish between these two possibilities.

Although genetically engineered cells often provide useful models for studying cancer, it is always important to confirm *in vitro* anticancer effects of a novel treatment in human cancer cells. Hence, the effects of (-)-agelastatin A were investigated in MDA-MB-231 and MDA-MB-435s human breast cancer cells, both of which are highly

metastatic. Previously, we have shown that only MDA-MB-435s cells overexpress OPN (Fig. 1B) and that MDA-MB-231 cells do not express detectable levels (data not shown). These results are concordant with other published studies (32). The ability of (-)-agelastatin A to repress OPN protein expression in MDA-MB-435s cells is in line with the data obtained for rat mammary cells.

Studies were also done to investigate how (-)-agelastatin A affects the OPN promoter (Fig. 1C). The data obtained were consistent with the Western blot analyses (Fig. 1A and B), which revealed that (-)-agelastatin A could increase Tcf-4 protein levels while reducing β-catenin and OPN. It is thought that Tcf-4 inhibits OPN transcription, whereas β-catenin coactivates it (3, 9). Thus, (-)-agelastatin A modulates key Wnt signaling effector molecules that can significantly influence OPN transcription and OPN-mediated neoplastic transformation. Further studies are currently under way to understand how (-)-agelastatin A down-regulates OPN and β-catenin using microarray techniques. Such methodology might shed valuable new light on why Tcf-4 levels increase when OPN and β-catenin are down-regulated by (-)-agelastatin A.

Uncontrolled cell growth is the hallmark of all cancers, including breast cancer (33–35). Compounds that can halt or significantly slow the growth of tumor cells may therefore serve as useful anticancer therapies. The data obtained in this study, on the effects of (-)-agelastatin A on cell growth, are consistent with previous findings that have shown that (-)-agelastatin A can inhibit tumor cell growth *in vitro* (13–15). Our results also show that despite contrasting OPN expression levels in benign Rama 37 and OPN-expressing metastatic C9 cells, (-)-agelastatin A affects their growth in a similar manner. Correspondingly, we have shown previously that no significant difference in growth rate is evident between parental Rama 37 and Rama 37 stably transfected by OPN (19). Similar growth-inhibitory effects with (-)-agelastatin A were also observed in MDA-MB-231 and MDA-MB-435s cells, although the MDA-MB-231 cells do not express OPN. Therefore, the cell growth-inhibitory effects of (-)-agelastatin A appear to be independent of OPN levels within the cells. Our comparisons of the growth of human cancer cells with rat mammary cells have indicated that (-)-agelastatin A is a more potent inhibitor of cell growth in human cancer cells. In rat mammary cells, at a concentration of 10 nmol/L, growth was similar to that of untreated cells (Fig. 2). However, in both MDA-MB-231 and MDA-MB-435s cell lines, growth was decreased at this concentration. Our study thus suggests that human cancer cells are more sensitive than rat cells to the effects of (-)-agelastatin A at low concentration. (-)-Agelastatin A induces growth inhibition through cell cycle arrest. As already shown, cells treated with (-)-agelastatin A accumulate in the G₂ phase of the cell cycle with a corresponding increase in cyclin B1 expression and a down-regulation of cyclin D1 and cyclin E expression. However, the mechanism by which (-)-agelastatin A causes this cell cycle arrest still needs to be elucidated.

Metastasis is a highly complex process where cancer cells move from a localized primary site to a distant secondary site via the blood or lymphatic systems of the host or through simple movement of the tumor cells within body cavities. The early genetic and phenotypic changes that accompany metastasis are typically complex (2, 32) but contribute significantly to the success with which most human cancers metastasize. The processes of metastasis require that local cell-cell interactions are disrupted, that blood and lymphatic vessels penetrate into the tissues that are to be colonized, that extravasion (or tumor cell escape) occurs from those vessels, and, finally, that migration, growth, adhesion, and colony formation all subsequently proceed. Adhesion molecules such as OPN play a fundamental role in allowing the processes of metastasis to occur; they also help direct where the secondary tumors form, as do local properties such as growth factor production, endothelial cell function, and the composition of the extracellular matrix.

The present study has shown that at concentrations as low as 10 nmol/L (-)-agelastatin A can inhibit OPN-mediated adhesion, colony formation, migration, and invasion in several important *in vitro* cellular models of metastasis. Of special note is the fact that despite (-)-agelastatin A inducing only a 10% reduction in OPN levels in these systems, this was sufficient to significantly attenuate the invasive potential of the cells studied. This indicates that (-)-agelastatin A can function as a powerful anti-invasive agent even at quite low drug concentrations.

Next, we used RNA interference to further confirm that (-)-agelastatin A modulates its effects on adhesion, migration, and invasion through the Wnt signaling pathway. Our data indicated that Tcf-4 siRNA could significantly increase OPN protein expression and was consistent with past work in our laboratory, which showed that Tcf-4 inhibition could enhance the expression of OPN (3). Interestingly, these siRNA down-regulatory effects on Tcf-4 could be reversed by treatment with (-)-agelastatin A, which significantly up-regulated or "rescued" Tcf-4 expression in Tcf-4 siRNA Rama 37 cells, which was in line with our previous findings. Naturally, however, the fact that (-)-agelastatin A could significantly "rescue" a previously siRNA silenced Tcf-4 gene in this system immediately raised questions about how it was capable of counteracting the effects of the siRNA and reversing the phenotype. The transfected expression vectors used for Tcf-4 siRNA place its expression under the control of the CMV promoter, which contains a major core of 11 and 3 recognition sequences for c-jun and Ets transcription factors (19), respectively. We hypothesize that (-)-agelastatin A might be inhibiting one or more of these transcription factors and so interfering with the expression of Tcf-4 siRNA. Of potential relevance here is the fact that c-jun is a direct target gene of the Wnt signaling pathway that is controlled by β -catenin (36, 37). It has also been established that the PEA-3 subfamily of Ets transcription factors cooperate with β -catenin, Lef-1, and c-jun to elicit transactivation from the matrilysin (38) and OPN (20) pro-

moters. It is entirely possible therefore that by down-regulating β -catenin expression, (-)-agelastatin A might be interfering with the functioning of c-jun or one or more Ets transcription factors that are coactivated by β -catenin rather like the ones just mentioned for matrilysin and OPN.

Another way in which (-)-agelastatin A might be reversing siRNA-induced Tcf-4 gene silencing might be through direct inhibition of the RNA-induced silencing complex endonuclease or the helicase or RNase that lie within the RNA-induced silencing complex, both of which are essential for mRNA cleavage and effective gene silencing to occur (39). A further possibility is that (-)-agelastatin A might be down-regulating the expression of one or more of the key protein components of the RNA-induced silencing complex endonuclease. Although outside the scope of the present study, future microarray work in our laboratory might shed some valuable light on how (-)-agelastatin A is overcoming these effects of siRNA Tcf-4 gene silencing. Notwithstanding our incomplete understanding of the origins of this phenotypic rescue, our collective data fundamentally support the earlier work that we have done, which shows that (-)-agelastatin A can significantly reduce the metastatic potential of cancer cells.

Targeting OPN and its Wnt coregulators with (-)-agelastatin A could significantly influence the metastatic spread of cancer, including breast cancer. The synthetic (-)-agelastatin A (15, 16) that we used in this study was shown to inhibit OPN protein expression in part through its potent modulatory effects on two components of the Wnt signaling pathway, that is, β -catenin and Tcf-4, although precisely how (-)-agelastatin A modulates these two Wnt effector proteins still remains unclear. Genes already known or suspected to have a down-regulatory effect on β -catenin-mediated signaling include APC, axin (40), conductin/axil, δ -TRCP (41), DAPPER (42), NOTCH1 (43), PPA2 (44), presenilin (40), TLE/Groucho, TSC1/2 (45), tuberlin-hamartin (46), thyroid hormone receptor (47), and p300/CRBP (48). Genes thus far implicated in regulating Tcf-4 levels include the Nemo-like kinase ring finger protein, NARF (which regulates ubiquitination and degradation of Tcf/Lef), and the Fgf15 gene. It might well be that some of these β -catenin and Tcf-4 modulatory genes are themselves being selectively or multiply targeted by (-)-agelastatin A. Future microarray work on (-)-agelastatin A and its analogues will help determine this. Microarray technologies might also allow us to identify novel (-)-agelastatin A-dependent transcripts that are playing a role in regulating OPN expression and OPN-mediated cell adhesion and migration.

Before departing from this discussion, it is perhaps pertinent to point out that epidermal growth factor can induce OPN gene expression in cancer (49). Activation of the epidermal growth factor receptor has likewise recently been shown to activate β -catenin expression in liver carcinomas (50). It is therefore tempting to also speculate that the epidermal growth factor receptor might be a possible target for (-)-agelastatin A. Future studies in this area might give valuable insights into such a hypothesis.

The fact that (-)-agelastatin A can potently down-regulate β -catenin expression within metastatic cancer cell lines (at 10 nmol/L concentration) means that not only can it halt tumor cell growth and proliferation but also it can combat metastasis, because it can simultaneously down-regulate key proteins involved in metastatic spread that are modulated by OPN. Moreover, because β -catenin controls transcription from the multidrug resistance 1 gene (51–53), this means that (-)-agelastatin A might also be far less susceptible to encountering drug resistance problems (unlike many existing antitumor drugs; ref. 53); potentially, it might sensitize tumors to effects of existing drugs and thus potentially be useful both as a single agent and as a combination therapy.

Because there are currently no good broad-spectrum antimetastatic drugs that have proven effectiveness (33), the introduction of a powerful new antimetastatic drug would constitute a major medical breakthrough. The clinical introduction of a dual-action, broad-spectrum, antitumor drug that could simultaneously inhibit cancer cell growth and function as a potent antimetastatic drug would be of even greater significance. In conclusion, our study has shown that (-)-agelastatin A functions as potentially powerful antimetastatic drug through at least two different mechanisms: as an inhibitor of OPN-mediated malignant transformation through its inhibitory effects on Wnt signaling and also through its prevention of cell cycle progression.

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