

Cyclopamine inhibition of human breast cancer cell growth independent of *Smoothened* (*Smo*)

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Abstract Altered hedgehog signaling is implicated in the development of approximately 20–25% of all cancers, especially those of soft tissues. Genetic evidence in mice as well as immunolocalization studies in human breast cancer specimens suggest that deregulated hedgehog signaling may contribute to breast cancer development. Indeed, two recent studies demonstrated that anchorage-dependent growth of some human breast cancer cell lines is impaired by cyclopamine, a potent hedgehog signaling antagonist targeting the *Smoothened* (SMO) protein. However, specificity of cyclopamine at the dosage required for growth inhibition ($\geq 10 \mu\text{M}$) remained an open question. In this paper we demonstrate that hedgehog signaling antagonists, including cyclopamine, and a second compound, CUR0199691, can inhibit growth of estrogen receptor (ER)-positive and ER-negative tumorigenic breast cancer cells at elevated doses. However, our results indicate that, for most breast cancer cell lines, growth inhibition by these compounds can be independent of detectable *Smo* gene expression. Rather, our results suggest that cyclopamine and CUR0199691 have unique secondary molecular targets at the dosages required for growth inhibition that are unrelated to hedgehog signaling.

Keywords Mammary cancer · Hedgehog signaling · Breast cancer treatment · Targeted therapy

Introduction

Genes in the mammalian hedgehog signal transduction network are key regulators of the development of most organs in the body, including the mammary gland. In these organs, hedgehog network genes controls proliferation, cell fate, and patterning, as well as stem/progenitor cell maintenance and self-renewal [1–5]. Altered hedgehog signaling is implicated in the development of approximately 20–25% of all cancers [6], especially those of soft tissues. With respect to human breast disease, data are accumulating to implicate altered hedgehog signaling in early mammary cancer development or disease progression, and to suggest that hedgehog signaling antagonists may be viable therapeutic or preventive agents [5, 7–13]. However, the specificity of hedgehog signaling antagonists at the elevated dosages required for inhibition of breast cancer cell growth remains an open question.

In mammals, “canonical” *hedgehog* signaling [2–4, 14] involves a signaling cell expressing a member of the hedgehog family of secreted ligands (*Sonic Hedgehog* (SHH), *Indian Hedgehog* (IHH), or *Desert Hedgehog* (DHH)), and a responding cell expressing one or more *Patched* family hedgehog receptors (*Patched-1* (PTCH1) and *Patched-2* (PTCH2)). In the absence of ligand, PTCH1 and PTCH2 can inhibit downstream signaling by antagonizing the function of the *Smoothened* (SMO) transmembrane effector protein. Under these conditions, expression of *hedgehog* target genes is inhibited by repressor forms of one or more members of the *Gli* family of transcription factors (GLI2 or GLI3). In the presence of ligand, PTCH1 releases

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inhibition of SMO, which leads to induction of target genes by transcriptional activator forms of *Gli* transcription factors (GLI1, GLI2, or GLI3). In addition to this “canonical” pathway, evidence for “noncanonical” hedgehog signaling has emerged recently [15–18].

In human breast cancer, we and others have demonstrated that expression of some hedgehog network genes is altered in clinical samples of human breast cancers, as well as in breast cancer cell lines [9–12], with the consensus finding that PTCH1 expression is reduced, or lost, in about 50% of all breast cancers, while SMO, the sole known effector of activated signaling, is ectopically expressed in ~70% of ductal carcinoma in situ (DCIS) and ~30% of invasive breast cancer (IBC). In mutational and array CGH analysis, *Ptch1* mutations, polymorphisms, and genomic losses have been identified in a subset of human breast cancers [7, 8, 13]. All of these data are consistent with the possibility of active, *Smo*-mediated hedgehog signaling in a significant proportion of human breast lesions, particularly early lesions.

A number of hedgehog signaling antagonists have been characterized [19–22]. These compounds include a group of plant-derived steroidal alkaloids first identified as potent teratogens in sheep. Structurally related compounds, including veratramine and tomatidine, are not teratogenic, but neither are they biologically inert. In addition to naturally occurring antagonists, several other hedgehog signaling antagonists have been synthesized, including CUR0199691 [20, 23]. These hedgehog signaling antagonists are thought to act by direct binding to SMO thereby inhibiting all downstream canonical signaling. However, cyclopamine is also known to decrease phosphorylation of Akt, reduce expression of cdk2, and induce p27 protein expression via an unknown mechanism [24].

In most cell lines used to examine the molecular mechanism of hedgehog signaling, SMO antagonists have IC₅₀ values in the 300 nM range or below [20, 21, 25–27] as measured by molecular readouts for network activity, including reduction of *Ptch1*, *Gli1* and *Hhip* expression (generally considered “universal” targets induced by activated hedgehog signaling), and by reduction in reporter gene expression GLI-dependent reporter assays. Both cyclopamine and CUR0199691 have been used successfully in vivo to treat hedgehog network-induced cancers [28–33]. Mice treated with these agents show little evidence of adverse side effects.

Recently, two groups have shown that cyclopamine can inhibit growth of a subset of breast cell lines in vitro at doses of around 10 μM and above [9, 10]. Cyclopamine was shown to inhibit proliferation and to induce apoptosis, as well as to inhibit expression of a *Gli*-dependent luciferase reporter in sensitive cell lines [10]. These data suggested that hedgehog signaling may be active in a

subset of human breast cancer cell lines, and that SMO antagonists can inhibit breast cancer growth. However, both studies required relatively high doses of cyclopamine to affect growth inhibition. Further, in the study by Kubo et al. [9] expression of *Smo*, the molecular target of cyclopamine, was not demonstrated. Conversely, in the study by Mukherjee, *Smo* mRNA was detected in all cell lines tested, generally at low levels, regardless of their sensitivity or resistance to cyclopamine treatment. Thus, as pointed out by Mukherjee et al., the specificity of cyclopamine at doses required for growth inhibition of human breast cancer cells remained an open question [10, 32]. Testing of these compounds in breast cancer cell lines that do not express detectable *Smo* is required to separate *Smo*-mediated effects from potential off-target growth inhibitory effects.

In this study, we evaluated the molecular and growth responses of a panel of *Smo*-positive and *Smo*-negative human breast cancer cell lines to treatment with recombinant SHH ligand, as well as to two antagonists of SMO-mediated hedgehog signaling, cyclopamine and CUR0199691. Consistent with previously published data, cyclopamine (10–20 μM) was able to inhibit tumorigenic cell growth. In contrast, cyclopamine did not inhibit growth of immortalized, but non-tumorigenic breast cell lines. However, in our hands, growth inhibition did not correlate with the detectable expression of *Smo*, or with the ability of the cells to respond molecularly to SHH treatment. Further, cyclopamine sensitivity could not be competed by co-administration of SHH. Finally, treatment with a second SMO antagonist, CUR0199691, showed a pattern of cell type-specific activity different from that of cyclopamine, and at doses well above those required to inhibit hedgehog signaling.

Taken together, these data indicate that growth inhibition of many human breast cancer cell lines by *Smo* antagonists can be independent of their effects on SMO-mediated hedgehog signaling, and suggest that cyclopamine and CUR0199691 have unique secondary molecular targets at elevated dosages. Intriguingly, in the case of cyclopamine, and for the set of cell lines we tested, there appears to be selectivity for inhibiting growth of tumorigenic, but not non-tumorigenic breast cancer cell lines.

Materials and methods

Human breast cancer cell lines and culture conditions

MCF7, BT474, T47D (estrogen receptor positive (ER+), tumorigenic) MDA-MB-231, and SKBR3 (estrogen receptor negative (ER–), tumorigenic), and MCF10A, MCF12A (ER–, immortalized, non-tumorigenic) human

breast cancer cell lines were obtained from the American Type Culture Collection (ATCC). Tumorigenic cell lines were maintained in Minimal Essential Medium (MEM), 0.01 mg/ml bovine insulin, and 10% fetal calf serum. MCF10A and MCF12 cells were maintained in 1:1 Dulbecco's Modified Eagles Medium:F12 (DMEM/F12), 15 mM HEPES, 2 mM L-glutamine (Invitrogen), 5% horse serum, 20 ng/ml EGF, 100 µg/ml cholera toxin and 5 ng/ml hydrocortisone. All cultures were grown at 37°C, with 5% CO₂ in air.

Hedgehog signaling inhibitors and recombinant SHH-N ligand

Cyclopamine was a generous gift from Dr. William Gaffield (USDA, retired), or was purchased from Toronto Research Chemicals (Ontario, Canada). Cyclopamine was dissolved in 100% ethanol for stock solutions (10 mM) and remained active for at least 6 months when stored at 4°C. CUR0199691 was a generous gift from Curis Inc. (Cambridge MA) through an agreement with Genentech Inc. (South San Francisco CA), and was dissolved in 100% ethanol for stock solutions. Tomatidine (Sigma Chemical) was dissolved in methanol:chloroform (1:1) for stock solutions (10 mM) and prepared fresh for each experiment due to progressively deteriorating behavior of stock solutions after their initial use. Purified, dual lipid-modified, recombinant SHH-N ligand was kindly provided by Curis Inc. (Curis Inc., Cambridge MA).

Anchorage-dependent dose-response growth assays

With the exception of T47D and BT474, which were plated at 1,000 cells/well, all breast cancer cell lines were plated 500 cells/well in triplicate wells of 96-well dishes. Medium was replaced on day 1 of culture by fresh medium at which time cells were either left untreated, or were treated with either SHH-N (1 and 10 ng/ml), tomatidine (5, 10, and 20 µM), cyclopamine (5, 10, and 20 µM), or CUR0199691 (3 and 6 µM). Medium was replaced on treatment day 4. Cell proliferation assays were performed on treatment days 1, 4, and 7 using reduction of (3-[4,5-Dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt as an indicator (MTS assay, Promega). Plates were then incubated at 37°C for 2.5 hours and read on a 96-well plate reader at 550 nm.

Agonist-antagonist competition assays

All cell lines were plated as for the continuous exposure dose-response assay and treated as follows: untreated, SHH-N alone (1 or 10 ng/ml), Cyclopamine alone (1, 5, 10, or 20 µM), Tomatidine alone (1, 5, 10, or 20 µM),

Cyclopamine + SHH-N, and Tomatidine + SHH-N. Medium was replaced on treatment day 4 by fresh medium containing drug. MTS cell proliferation assays were performed on treatment days 1, 4 and 7. Data were plotted and analyzed as for the dose response assays above.

Anchorage-independent growth in soft agar

MCF7 cells were plated in 6-well dishes over a bottom layer consisting of 2 ml of medium with 0.7% agarose. 5,000 cells per well were plated in a top layer consisting of 4 ml of medium with 0.35% agarose (SeaPlaque agarose, BioWhittaker Molecular Applications). MDA-MB-231 cells were plated over a bottom layer consisting of 1.5 ml of medium with 0.6% agarose. 20,000 cells per well were plated in a top layer consisting of 4 ml of medium with 0.3% agarose. Cells were allowed to grow for 2 days in the absence of drug at 37°C in a 5% CO₂ humidified incubator. Cells were then treated with 0, 10, 20 or 30 µM cyclopamine or tomatidine (final concentration) in 2 ml fresh medium with replacement at one week. After 14 days (MCF7), or 21 days (MDA-MB-231), colonies were stained using the MTT assay for reduction of (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (Sigma Aldrich). The cells were incubated for 4 h at 37°C in a 5% CO₂ humidified incubator and placed at 4°C overnight to allow complete color change. Plates were photographed using a stereomicroscope, and colonies counted using QuantityOne software.

Quantitative RT-PCR

All gene expression assays were conducted on day 4 of treatment. Total RNA was extracted from each well individually using Trizol reagent (Invitrogen) according to manufacturer's instructions. Changes in hedgehog network gene expression in response to treatment were evaluated by TaqMan quantitative RT-PCR using Assay-On-Demand primer/probe sets (ABI) for *Shh*, *Ihh*, *Dhh*, *Ptch1*, *Ptch2*, *Smo* (three unique primer/probe sets), *Hhip*, *Gli1*, *Gli2*, and *Gli3*. CT values above 35 indicate expression levels at, or near, the limit of detection for the method (Applied Biosystems Application Note). Thus, genes with average CT values above 37 were designated as undetectable. Each experiment was performed using triplicate reactions, with triplicate RNA samples for each treatment, from at least three independent experiments.

Additional controls for specific detection of Human *Smo* mRNA included RNA derived from transgenic mice expressing a human *Smo* transgene (*MMTV-SmoM2*) relative to non-transgenic control mice expressing no human genes, as published previously [12].

Flow cytometry

For evaluation of apoptosis in response to cyclopamine, cells ($1 \times 10^6/\text{ml}$) were washed in Annexin V binding buffer (34), resuspended in 50 μl of the same buffer and then incubated in the dark with 1 μl of FITC-conjugated annexin-V (Biosource, CA) on ice for 30 min. After incubation cells were washed and resuspended in the same buffer. Flow cytometric analysis was performed using Epics XL-MCL flow cytometer (Beckman-Coulter, CA). The amount of FITC-annexin V was determined by measuring its fluorescence relative to unstained negative control cells.

Cholesterol accumulation assay

Intracellular cholesterol accumulation was evaluated by filipin staining [35, 36]. Cells were fixed for 1 h in 10% neutral buffered formalin and stained with 125 $\mu\text{g}/\text{ml}$ solution of filipin in PBS and 0.5 ml of DMSO and incubated at 37°C in a 5% CO_2 humidified incubator for 3 h in the dark. The coverslips were washed in PBS and mounted in medium containing propidium iodide as a nuclear counterstain (Vectashield, Vector Laboratories, Inc.). Fluorescence microscopy for filipin staining was performed using UV excitation and a standard DAPI detection filter set on a Nikon E1000 epifluorescence microscope. All images were exposure matched based on exposure tests using a separate set of control cells due to the rapid photobleaching of cholesterol-bound filipin.

Statistical analysis

Five different types of data were analyzed in this study. Anchorage-dependent dose-response growth assays and agonist-antagonist competition assays were analyzed using general linear models. The log transformed absorbance values were used as response variables. Treatment dose responses were modeled over time. Contrasts were used to examine differences between untreated controls and treatments. Mean and 95% CIs were back-transformed and plotted in the original measurement units of absorbance for each day and treatment for all the experiments.

For proliferation (Ki67 expression) and apoptosis (Annexin-V expression) assays, mean percentage of cells expressing Ki67 or Annexin-V in response to treatment were analyzed with arcsine square root transformation. Overall comparisons across all treatment groups were performed separately for MCF7, MDA-MB-231 and MCF10A using a one-way ANOVA. When strong evidence of a difference across treatment groups was indicated, additional comparisons of treatment to control were carried out using contrast statements within the ANOVA. Mean

and 95% CIs were back-transformed and plotted in the original measurement units for each treatment for all the experiments.

Quantified colony formation in anchorage-independent soft agar assays in response to treatment with cyclopamine and tomatidine was normalized to untreated control. A one-way ANOVA was performed separately for MCF7 and MDA-MB-231 to test a difference in colony formation across all treatment groups. A similar analysis strategy as described above for Ki67 and Annexin V expression was applied. Data were plotted as mean percent of control with one standard deviation.

Quantitative RT-PCR data were analyzed using the $\Delta\Delta\text{CT}$ method [37] in which expression of β -actin was used to normalize the relative expression of each gene within each cell line. For comparative analyses across all cell lines, expression of each individual gene in brain mRNA was then used as the calibrator by which the cell lines could be normalized with one another. This method allowed for comparison of relative expression of hedgehog network genes across the six cell lines examined in detail. Testis mRNA was also used as a positive control for hedgehog network gene expression. Hedgehog network gene expression in human breast cancer cell lines in response to treatment was compared with untreated cells using a two-sample t-test. *P*-values of 0.05 or less were deemed statistically significant.

Results

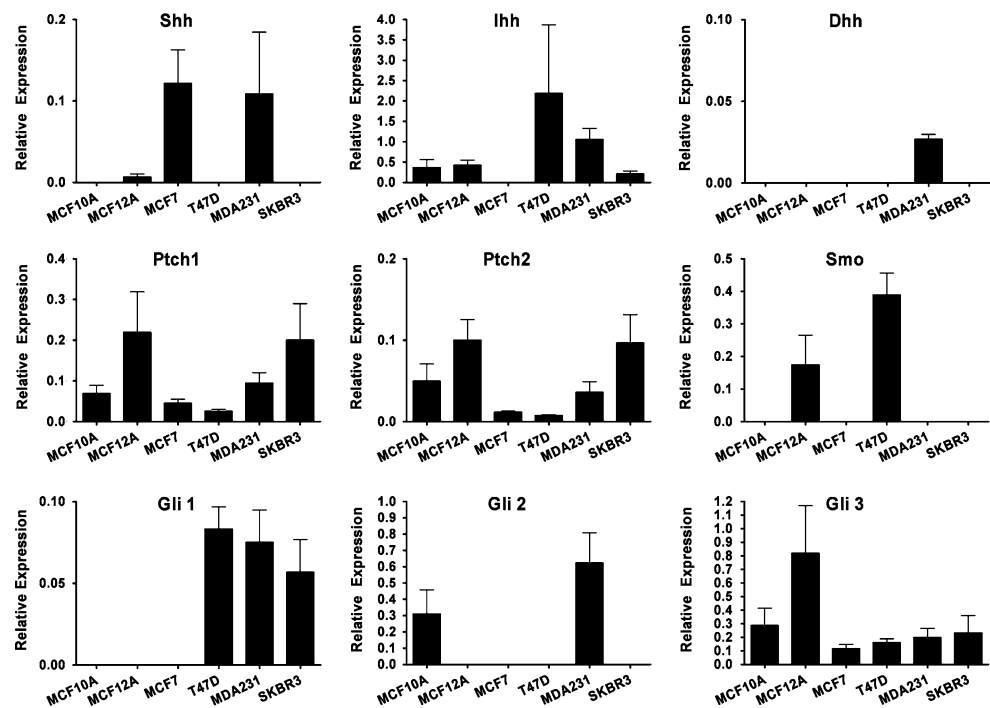
Expression of hedgehog network genes in untreated breast cancer cell lines

Because previous work showed that the dosages of cyclopamine required for growth inhibition were 10- to 100-fold those required in other cell lines to block canonical hedgehog signaling, we first wished to verify relative expression of hedgehog network genes, particularly *Smo*, the high-affinity target for cyclopamine, using our collection of human breast cancer cell lines (Fig. 1).

Using human brain and testis mRNA as a positive controls, *Shh* mRNA was detected only in MCF7 and MDA231 with lower levels detectable in MCF12A. In contrast, *Ihh* was detectable in all cell lines tested except MCF7, with T47D showing expression levels 2.4-fold that observed in brain. *Dhh* was only detectable in MDA-MB-231 cells. Both *Ptch1* and *Ptch2* mRNAs were detected in all cell lines, and appeared to be coordinately expressed.

In our breast epithelial cell line collection, only T47D and MCF12A showed detectable *Smo* expression. Failure to detect *Smo* was confirmed using two additional primer/probe sets, including the identical primer/probe set used in

Fig. 1 Quantitative RT-PCR (Q-PCR) using untreated cells comparing hedgehog network gene expression across all cell lines tested. Data were analyzed using the $\Delta\Delta\text{CT}$ method in which expression of β -actin was used to normalize the relative expression of each gene within each cell line. Expression of each individual gene in brain mRNA was then used as the calibrator by which the cell lines could be normalized with one another for comparison of relative expression of hedgehog network genes across all cell lines. Data are expressed as the average quantification relative to expression in brain mRNA, with one standard error of the mean



the Mukherjee study [10] (Kindly provided by A. Frost), using RNA derived from MCF10A, MCF7, and MDA-MB-231 cells.

Of the three *Gli* genes, only *Gli3* was detectable in all cell lines, with both *Gli1* and *Gli2* expression completely lacking in MCF12A and MCF7, despite low level expression of *Smo* in MCF12A (Fig. 1). *Hhip*, generally considered a “universal target” for activated hedgehog signaling, was detectable at low levels only in MCF10A and MDA-MB-231 cells (Fig. 1, supplemental).

Breast cancer cell lines show varied responses to SHH ligand

Our gene expression screen suggested that, because only T47D and MCF12A expressed *Smo* mRNA, and all known hedgehog signaling is mediated by *Smo*, only these two cell lines should be capable of responding to hedgehog ligand treatment in a canonical fashion. If so, we expected to observe induction of hedgehog network gene expression (*Ptch1*, *Ptch2*, *Gli1*, *Gli2*, or *Hhip* as autoregulated “canonical” targets), growth stimulation, or both, in response to SHH-N ligand treatment.

Of the six cell lines tested, and consistent with cell line-specific expression of *Smo* mRNA, only T47D showed induction of other hedgehog network genes in a manner consistent with the presence of canonical hedgehog signaling at 10 ng/ml (Fig. 2a). Two lines, MCF7 and MCF10A, which did not express *Smo* mRNA, showed no measurable response to SHH-N (Fig. 2b and c, respectively). In contrast,

three lines (MCF12A, SKBR3 and MDA-MB-231) showed highly variable down-regulation of network genes in response to treatment (Fig. 2d–f, respectively). However, because of the variability, these changes were not statistically significant. Failure to detect network activation in MCF12A was consistent with the lack of detectable *Gli1* and *Gli2* expression, with elevated expression of both *Ptch1* and *Ptch2* relative to all other cell lines used, as well as with elevated expression of *Gli3*. None of the cell lines were growth-stimulated under these conditions (Fig. 3a–f).

Tumorigenic and non-tumorigenic breast epithelial cell lines show differential growth responses to high dose cyclopamine

Previous studies demonstrated that growth of selected breast cancer cells could be slowed or prevented by treatment with cyclopamine at $\sim 10 \mu\text{M}$ and above. Because only T47D expressed readily detectable *Smo* and responded to SHH-N treatment, we predicted that, only T47D should be growth-inhibited by SMO antagonists if the effect of SMO antagonists was specific. To test this prediction, we generated dose response curves for exposure to cyclopamine (0–20 μM) versus the negative control compound tomatidine (0–20 μM).

In our hands, the estrogen receptor positive (ER+) cell line MCF7 (Fig. 4a) was affected significantly at 10 and 20 μM cyclopamine ($P = 0.026$ and $P < 0.0001$, respectively). BT474 showed significant dose-dependent sensitivity

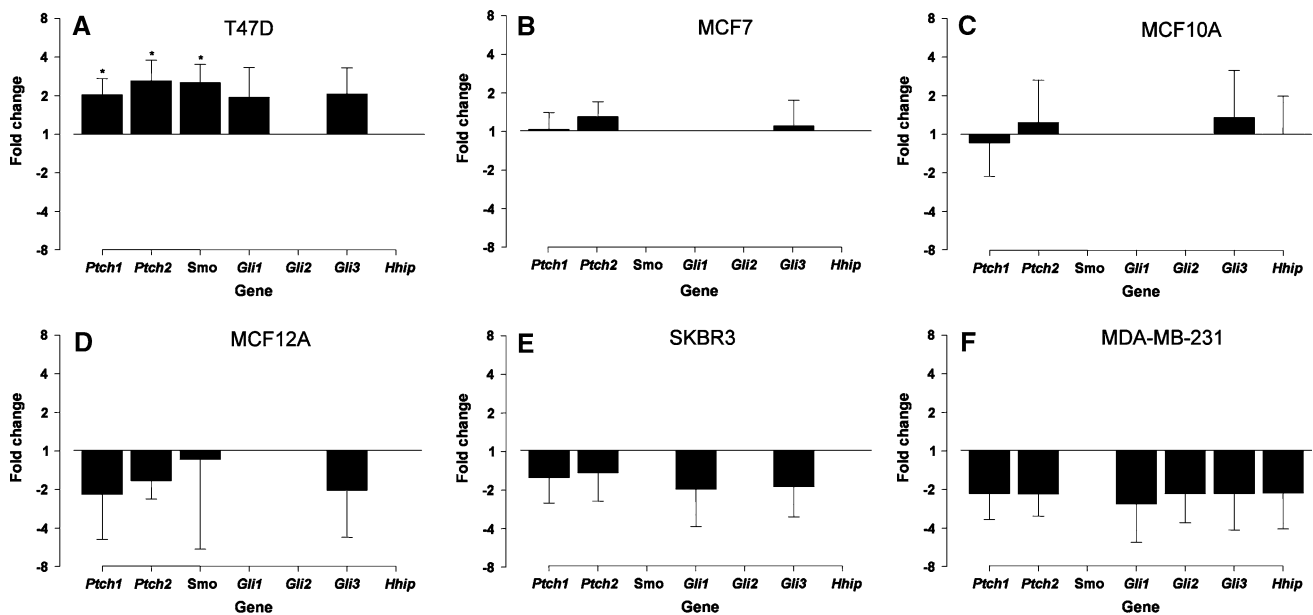


Fig. 2 Hedgehog network gene expression in human breast cancer cell lines in response to 10 ng/ml SHH-N ligand. Untreated values for each gene were normalized to “1”. Data are expressed as fold change

relative to untreated cells, with one standard error. Statistically significant changes in gene expression are denoted by an asterisk (*) (two-sample t-test)

to cyclopamine. However, BT474 also showed significant dose-dependent sensitivity to tomatidine at the 10 μ M and 20 μ M dosages (data not shown). Thus, BT474 cells were not studied further. T47D (Fig. 4b) showed significant growth inhibition only at 20 μ M ($P < 0.0001$). Neither MCF7 nor T47D showed sensitivity to tomatidine (Fig. 4a and b, respectively).

Similar to the ER+ cell lines, and despite lack of detectable *Smo* expression, both ER- cell lines, MDA-MB-231 (Fig. 4c) and SKBR3 (Fig. 4d) showed sensitivity to cyclopamine, with MDA-MB-231 showing significant sensitivity only at 20 μ M ($P < 0.0001$), and SKBR3 showing sensitivity at both 10 μ M and 20 μ M ($P = 0.05$ and $P < 0.0001$, respectively). Again, neither MDA-MB-231 nor SKBR3 showed sensitivity to tomatidine (Fig. 4c and d, respectively).

In contrast to the tumorigenic cell lines, and despite detectable *Smo* expression in MCF12A cells, both MCF10A cells (Fig. 4e) and MCF12A cells (Fig. 4f) were insensitive to cyclopamine at 10 μ M, with MCF10A showing significant sensitivity at 20 μ M ($P = 0.007$) due to a decrease in cell number on day 4 of treatment only. However, overall, growth curves with cyclopamine were similar to those for tomatidine treatment. To rule out an effect of different growth medium on cyclopamine sensitivity of transformed cell lines, dose-response curves were also generated for MCF7 and MDA-MB-231 in the same medium used for assays of MCF10A and MCF12A. Medium composition had no effect on cyclopamine sensitivity of these two cell lines (data not shown).

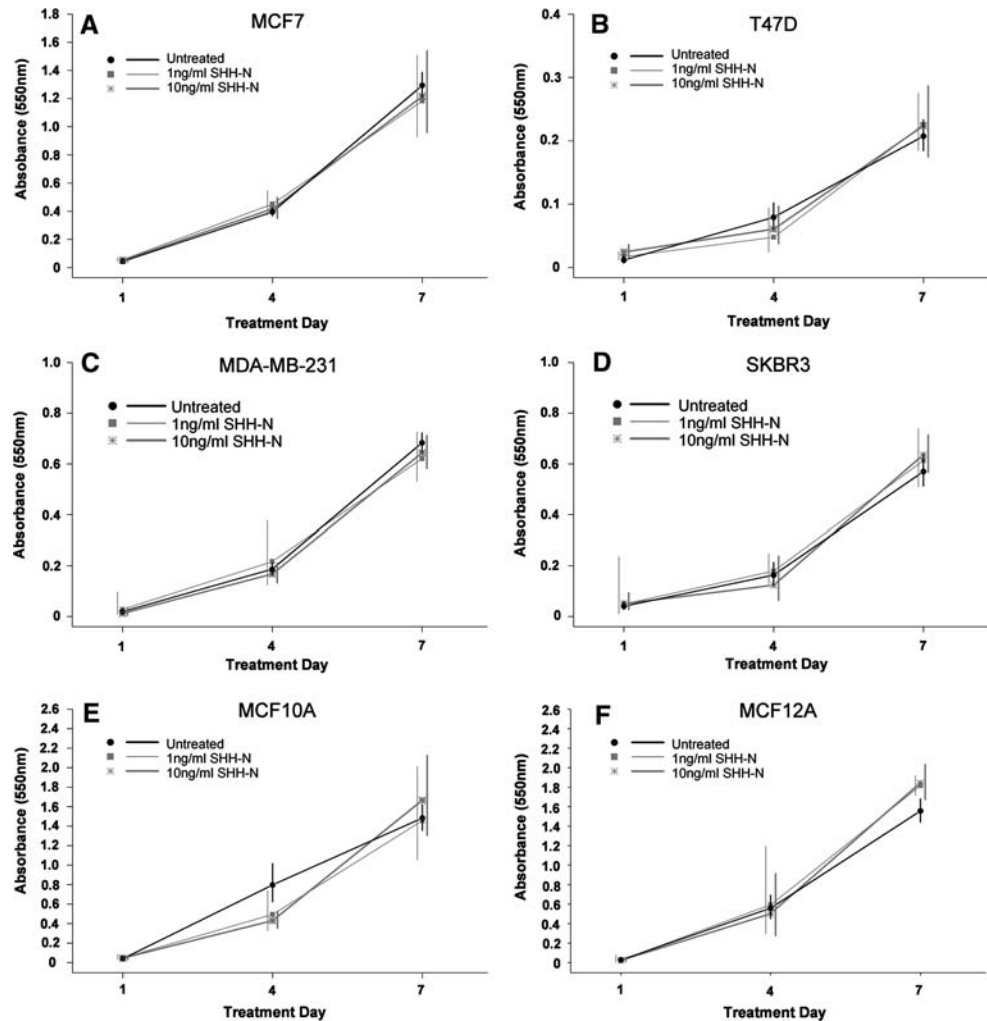
Cyclopamine inhibits proliferation and induces apoptosis in sensitive cell lines

To confirm consistency with previously published results, and to understand the mechanism(s) by which cyclopamine inhibits breast cancer cell growth, we assayed whether cyclopamine inhibited proliferation, induced apoptosis, or both using immunofluorescence analysis for expression of the proliferation marker Ki67, and flow cytometric analysis for expression of the apoptotic marker annexin V, in response to cyclopamine and tomatidine treatment.

Cyclopamine reduced Ki67 expression in MCF7 at the 10 μ M ($P = 0.015$) and 20 μ M ($P < 0.0001$) dosages (Fig. 5a), with a reduction in Ki67 expression also detectable at 10 and 20 μ M tomatidine ($P = 0.006$ and $P = 0.015$, respectively). Cyclopamine also reduced Ki67 expression in MDA-MB-231 significantly at the 20 μ M dose (Fig. 5b) ($P = 0.034$), with an unexplained, but consistent, increase in Ki67 expression at 10 μ M tomatidine ($P = 0.008$). Tomatidine showed a slight stimulatory effect on Ki67 expression at the 20 μ M ($P = 0.012$) in MCF10A (Fig. 5c).

Consistent with its inhibitory effect on anchorage-dependent growth in tumorigenic cell lines, cyclopamine induced apoptosis in both MCF7 (10 μ M, $P = 0.043$ and 20 μ M, $P < 0.0001$) and MDA-MB-231 (10 μ M, $P = 0.008$; 20 μ M, $P < 0.0001$) (Fig. 5d and e, respectively), with no significant effect on MCF10A cells (Fig. 5f). Tomatidine showed a modest effect on apoptosis in MCF7 at 20 μ M ($P = 0.046$), but showed no significant effect on either MDA-MB-231 or MCF10A.

Fig. 3 Dose response curves for growth of breast cancer cell lines in response to treatment with either 1 or 10 ng/ml SHH-N ligand relative to untreated cells. MTS assays were performed on treatment days 1, 4, and 7. Mean absorbance for each treatment and day was plotted with 95% confidence intervals



Cyclopamine inhibits anchorage-independent growth of tumorigenic breast cancer cells at high doses

To determine whether cyclopamine could also interfere with anchorage-independent breast cancer cell growth, we performed colony forming assays in soft agar using MCF7 (Fig. 2a, supplemental) and MDA-MB-231 (Fig. 2b, supplemental), treated with either cyclopamine (0–30 μ M) or tomatidine (0–30 μ M). For both cell lines we observed significant reduction in colony number at the 20 and 30 μ M doses relative to untreated controls ($P < 0.05$, two sample t-test), with no effect at the 10 μ M dose. Tomatidine showed no effect on colony formation except at 30 μ M in MCF7 cells.

Cyclopamine and tomatidine have effects on hedgehog network gene expression

To determine whether cyclopamine treatment was influencing hedgehog network gene expression differentially relative to tomatidine, and to determine whether growth

inhibition correlated with a particular change in hedgehog network gene expression, we performed quantitative RT-PCR (Q-PCR) for hedgehog network genes using RNA extracted from representative cell lines T47D, MCF7, MDA-MB-231 and MCF10A, that had been treated with 10 μ M cyclopamine or tomatidine for four days. Results are shown in Fig. 6.

Based upon our initial gene expression and SHH-N treatment analyses, of the four cell lines chosen for gene expression analysis in response to cyclopamine and tomatidine, we predicted that if hedgehog signaling were active in T47D cells under standard cell culture conditions, only T47D cells would show a significant response to cyclopamine by down-regulating hedgehog network gene expression.

Contrary to this expectation, T47D cells showed significant, or borderline significant, increases in gene expression in response to 10 μ M cyclopamine (Fig. 6a). Tomatidine showed no statistically significant change in network gene expression (Fig. 6a). Consistent with a lack of *Smo* expression, MCF7 and MCF10A cells showed no

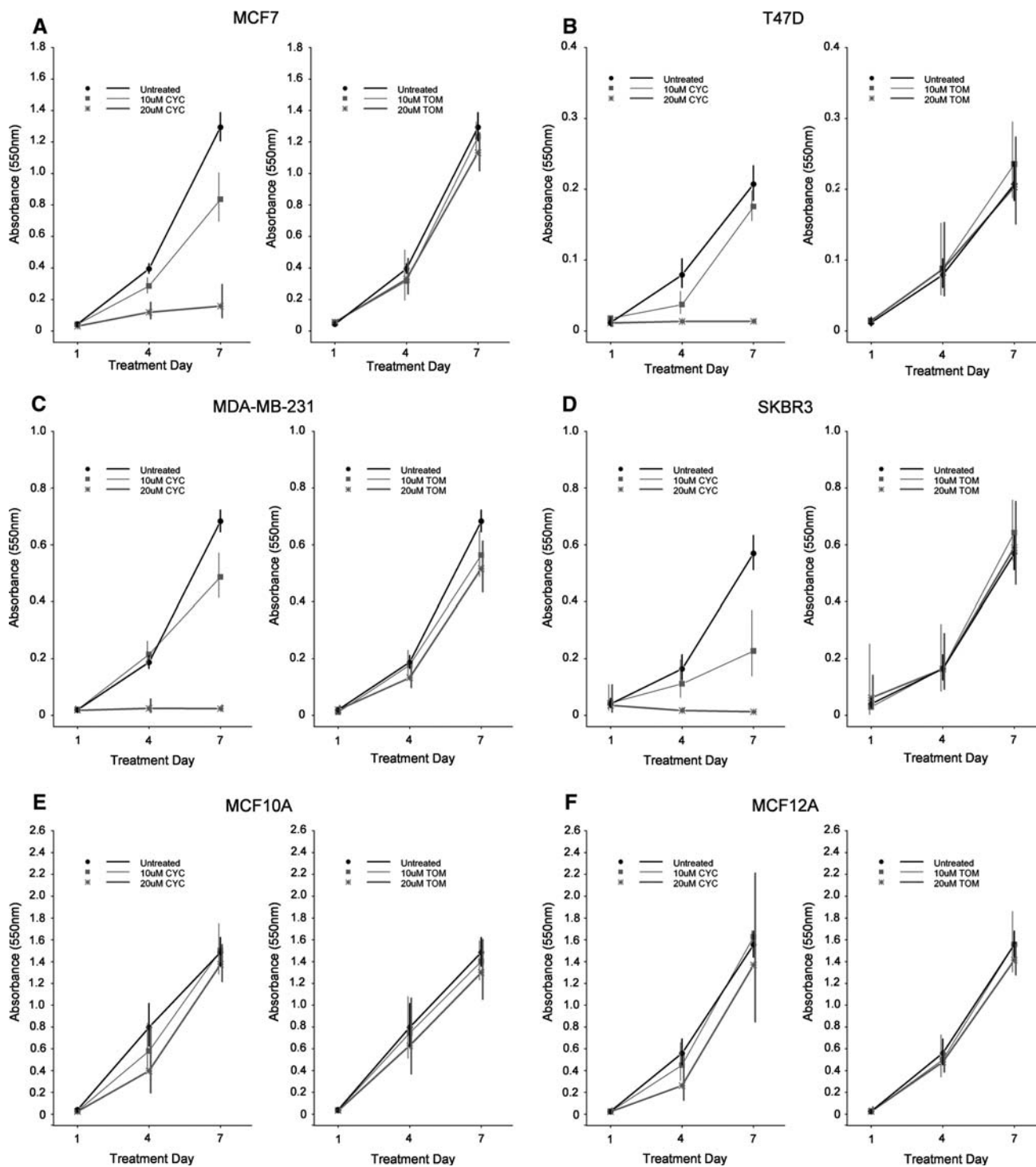


Fig. 4 Dose response curves for growth of breast cancer cell lines in response to treatment with either cyclopamine or tomatidine. MTS assays were performed on treatment days 1, 4, and 7. Mean absorbance for each treatment and day was plotted with 95% confidence intervals

significant response to either cyclopamine or tomatidine (Fig. 6b and c, respectively). Finally, MDA-MB-231 cells showed opposite, but statistically non-significant responses to cyclopamine (slight up-regulation) and tomatidine (slight down-regulation) (Fig. 6d). Thus, again consistent

with unique expression of *Smo* in T47D cells, only T47D cells responded significantly to cyclopamine treatment. However, the direction of response was opposite of that expected in that hedgehog network genes were invariably induced.

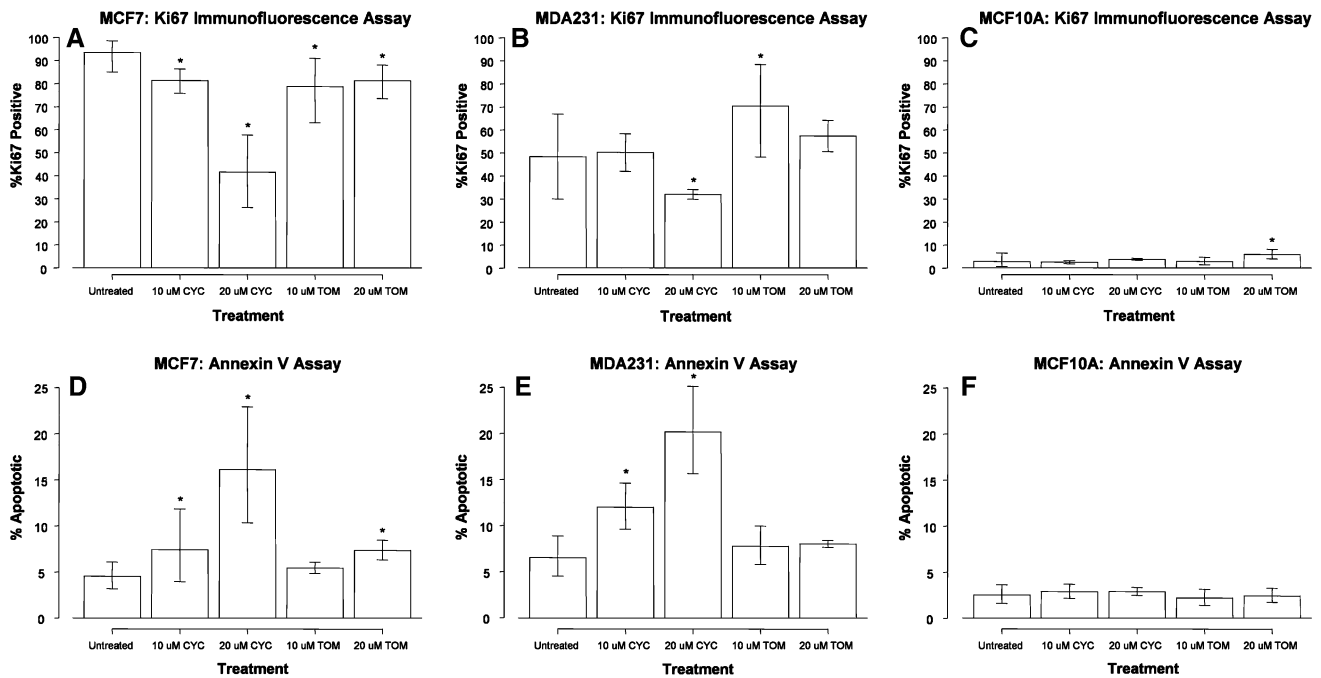


Fig. 5 Ki67 expression and apoptosis in cycloamine-sensitive and -resistant cell lines as a function of treatment. Proliferation is expressed as the percentage of Ki67+ cells determined by direct cell counts. Apoptosis is expressed as the percentage of Annexin V+ cells as determined by analytical flow cytometry. Results for three cell

lines, MCF7, MDA-MB-231, and MCF10A are shown. Mean percentage of cell expression Ki67 or Annexin V in response to treatment was plotted with 95% confidence intervals. Statistical significance relative to untreated control is denoted by an asterisk (*) (contrasts within a one-way ANOVA)

Effects of cycloamine on cholesterol trafficking are similar in sensitive and resistant cell lines, and are identical with those of tomatidine

At high doses, such as those required for growth inhibition in this and other studies, cycloamine and related compounds have known effects on cholesterol trafficking, with treatment leading to accumulation of cholesterol within the cell. This accumulation can be evaluated readily by fluorescence microscopy of cholesterol-bound filipin. It was possible that a proportion of the strong growth inhibitory effects we observed in tumorigenic cells could also be due to a differential effect of cycloamine versus tomatidine on cholesterol trafficking at higher doses relative to non-tumorigenic cells. To evaluate this possibility, we treated MCF7, MDA-MB-231, and MCF10A cells with increasing doses of cycloamine or tomatidine and stained with filipin. Results are depicted in Supplemental Fig. 3.

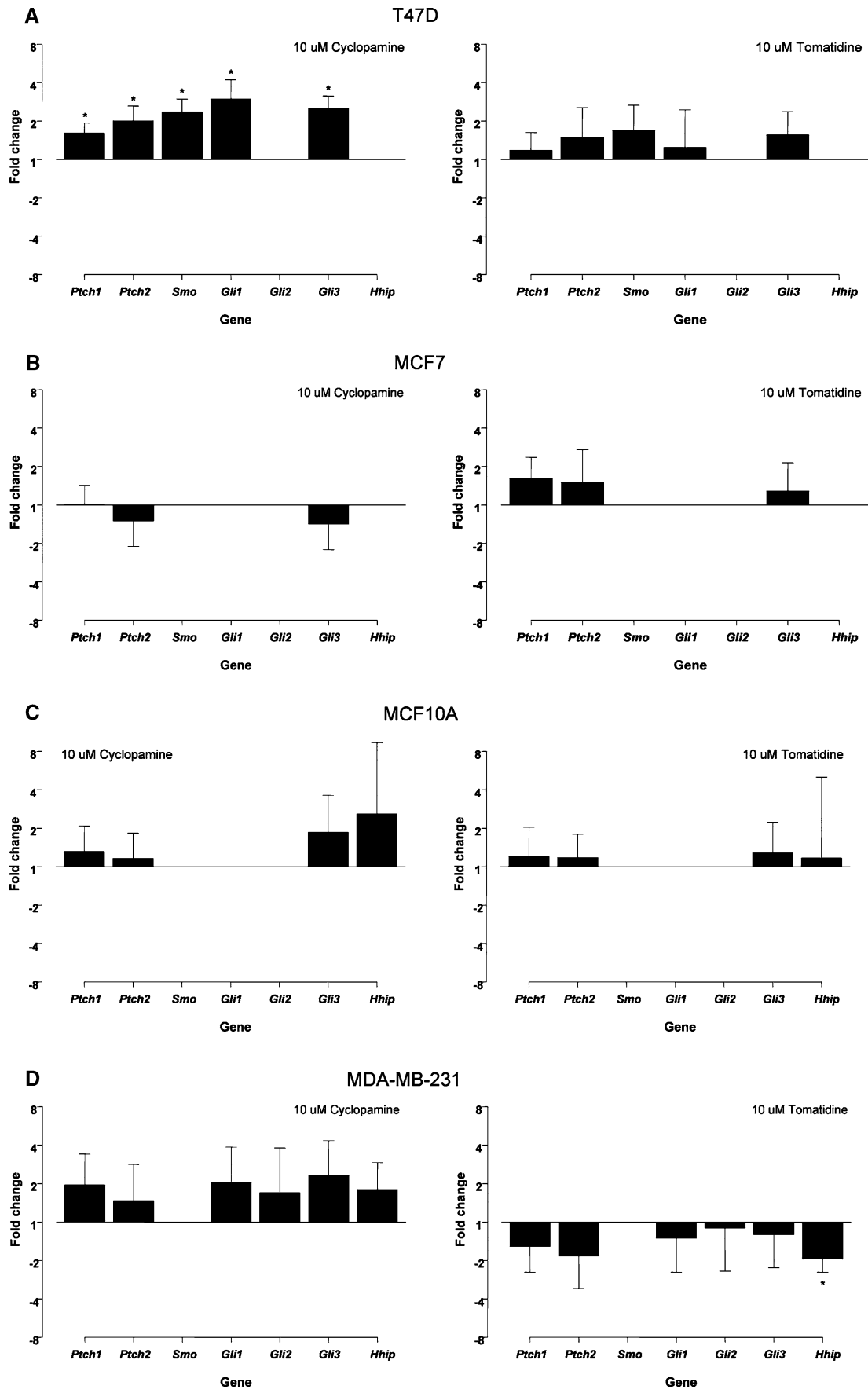
In untreated cells, filipin staining is observed primarily at the cell membrane (Fig. 3a, d, and g, Supplemental). However, in all three cell lines, we observed significant accumulation of cholesterol within the cells at doses as low as 10 μ M cycloamine (Fig. 3b, e, and h, Supplemental). Tomatidine also showed effects on cholesterol trafficking that were indistinguishable from those of cycloamine at doses as low as 10 μ M (Fig. 3c, f, and h, Supplemental).

Since we observed cholesterol accumulation in both sensitive and insensitive cell lines in response to treatment with either 10 μ M cycloamine or 10 μ M tomatidine, neither of which led to strong growth inhibition in these cells, cholesterol accumulation in response to cycloamine is not likely to be a significant influence on breast cancer cell growth.

SHH-N treatment shows interaction with cycloamine in T47D cells

Previous studies in other cell lines and explant cultures have demonstrated that cycloamine can block hedgehog signaling at doses between 200nM and 10 μ M [22, 27, 36, 38–40]. It was also shown that the interaction between cycloamine and SMO is regulated by PTCH1 function, with increased PTCH1 activity resulting in enhanced SMO:cycloamine complex formation that is reversible by treatment with recombinant SHH-N.

Dual lipid-modified SHH-N functions in the high picomolar to low nM concentration range [27, 36, 40]. Thus, if the growth inhibitory effects of cycloamine are specific for *Smo*-mediated hedgehog signaling, these effects should be ameliorated by competition with SHH-N ligand. Such competitive inhibition has been observed in mammosphere-formation assays using normal mammary epithelial



◀ **Fig. 6** Quantitative RT-PCR for hedgehog network gene expression as a function of treatment with 10 μ M cyclopamine or 10 μ M tomatidine relative to untreated cells using six cell lines. Untreated values for each gene were normalized to “1”. Data are expressed as fold change relative to untreated, with one standard error. Statistically significant changes in gene expression relative to untreated cells are denoted with an asterisk (*) (two-sample t-test)

cells from reduction mammoplasties [5], and in colorectal cancer cells in which induction of apoptosis by cyclopamine could be partially competed by treatment with conditioned medium containing recombinant SHH-N ligand [41].

To address this possibility, we conducted competition assays in which breast cancer cell lines were treated with cyclopamine alone (0–20 μ M), or with recombinant SHH-N ligand at either 1 ng/ml (0.62nM) or 10 ng/ml (6.2nM), either alone, or in competition with cyclopamine. Results are shown in Fig. 7. With the exception of T47D cells (Fig. 7b), competition with ligand did not rescue growth inhibition by cyclopamine for any other tumorigenic cell line tested (Fig. 7a, c, d), at any dose of cyclopamine. In fact, for MCF7, combined treatment with 20 μ M cyclopamine led to significantly enhanced growth inhibition at both 1 ng and 10 ng/ml SHH-N doses ($P \leq 0.001$) (Fig. 7a). In contrast with all other cell lines tested, and consistent with detectable *Smo* expression only in T47D cells, combined treatment of T47D cells with both SHH-N and cyclopamine led to enhanced growth relative to cyclopamine alone ($P < 0.001$) (Fig. 7b). Combined treatment had no effect on MDA-MB-231 or SKBR3 cells (Fig. 7c and d, respectively), nor on either of the two non-tumorigenic cell lines MCF10A and MCF12A (Fig. 7e and f, respectively).

Cyclopamine shows an unexpected interaction with SHH-N on hedgehog network gene expression in T47D cells

Because of the unexpected stimulatory effect of 10 μ M cyclopamine on hedgehog network gene expression in T47D cells (Fig. 6a), and the effect of combined SHH-N and cyclopamine treatment on T47D cells (Fig. 7b), we wished to determine whether cyclopamine and SHH-N showed additive or synergistic (multiplicative) effects on hedgehog network gene expression. Results are shown in Fig. 8. Low doses of cyclopamine alone showed a slight stimulatory effect on all genes tested, while 10 μ M cyclopamine alone and 10 ng/ml SHH-N alone each showed a statistically significant ~ 2 -fold stimulation. In combination treatment, 1 and 5 μ M cyclopamine did not show additive or synergistic effects on gene expression when co-administered with 10 ng/ml SHH-N. In contrast, 10 μ M cyclopamine with 10 ng/ml SHH-N was antagonistic, and

significantly inhibited network gene expression to levels at, or below untreated controls in many cases.

A second SMO antagonist, CUR0199691, shows different cell type selectivity from cyclopamine

To test further if the growth inhibitory effect of cyclopamine was due to specific antagonism of SMO-mediated hedgehog signaling, we used a second, more potent, hedgehog signaling antagonist CUR0199691. If growth inhibition by cyclopamine was due to specific effects on *Smo*-mediated signaling in the absence of detectable *Smo* expression, CUR0199691 should show growth inhibition greater than, or equal to, that conferred by cyclopamine. In addition, the cell-type specificity of inhibition should also be retained. We therefore generated dose-response curves for CUR0199691 using all seven cell lines. As with cyclopamine, MCF7, T47D, SKBR3 (Fig. 9a, b, and d, respectively), and BT474 (data not shown) cells showed growth inhibition in a dose-dependent manner. However, in contrast to their sensitivity to 20 μ M cyclopamine, MDA-MB-231 did not show significant inhibition in response to CUR0199691, even at 6 μ M (Fig. 9c). Also in contrast to their resistance to cyclopamine treatment, both MCF10A and MCF12A (Fig. 9e and f, respectively) were sensitive to treatment with 6 μ M CUR0199691 ($P = 0.0003$ and $P = 0.0018$, respectively). Because the cell lines affected by the two SMO inhibitors were not identical, these data suggested that the effects of both cyclopamine and CUR0199691 at growth-inhibitory doses were not due to specific effects on SMO protein function.

To determine whether CUR0199691 treatment influences hedgehog network gene expression, we performed quantitative RT-PCR (Q-PCR) for hedgehog network genes using RNA extracted from above cell lines that had been treated with CUR0199691.

Treatment with CUR0199691 showed no statistically significant change in network gene expression (Figs. 10 and 11), except MDA-MB-231 cells which showed a statistically significant increase of *Gli1* ($P = 0.02$) (Fig. 11).

Discussion

In this study, we confirm and extend previous observations that cyclopamine inhibits anchorage-dependent growth of both ER+ and ER– tumorigenic breast cancer cell lines [9, 10], and that cyclopamine functions both by inhibition of proliferation and induction of apoptosis in sensitive cell lines. In addition, and in contrast with the tumorigenic cell lines, we show that two immortalized, but non-tumorigenic cell lines are relatively insensitivity to cyclopamine at comparable doses. We also show that anchorage-

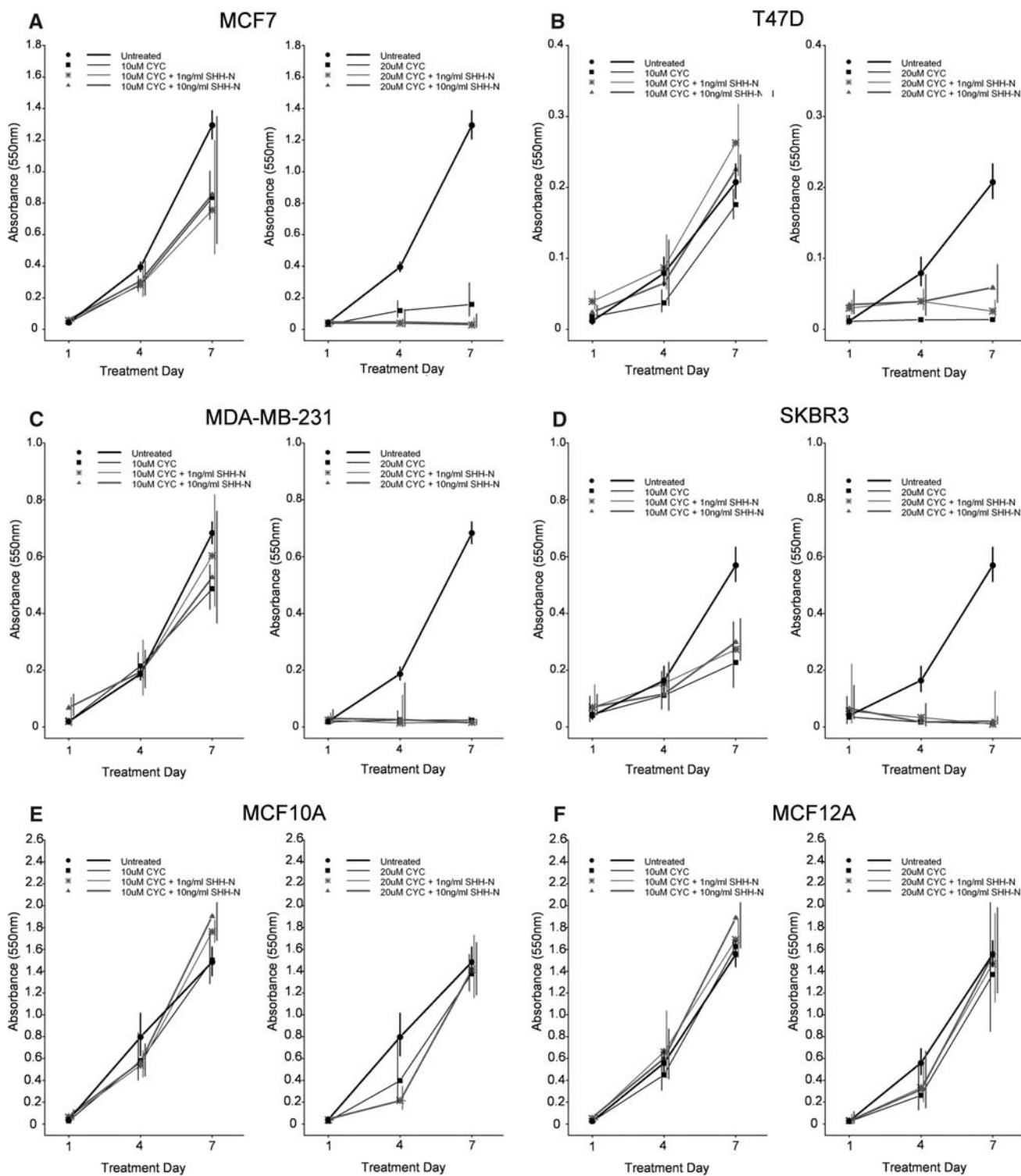


Fig. 7 Competition assays to determine whether the effect of cycloamine on cell growth could be reversed upon treatment with increasing concentrations of recombinant SHH-N. The left panel of each pair for each cell line shows results with 10 μM cycloamine.

The right panel of each pair shows results with 20 μM cycloamine. Mean absorbance for each treatment and day was plotted with 95% confidence intervals

independent growth is significantly impaired by cycloamine. However, in contrast to previous studies, we demonstrate that, of the cell lines tested, only T47D were

responsive to SHH-N treatment as evaluated by induction of “canonically” autoregulated hedgehog network gene expression. Further, we go on to demonstrate that the

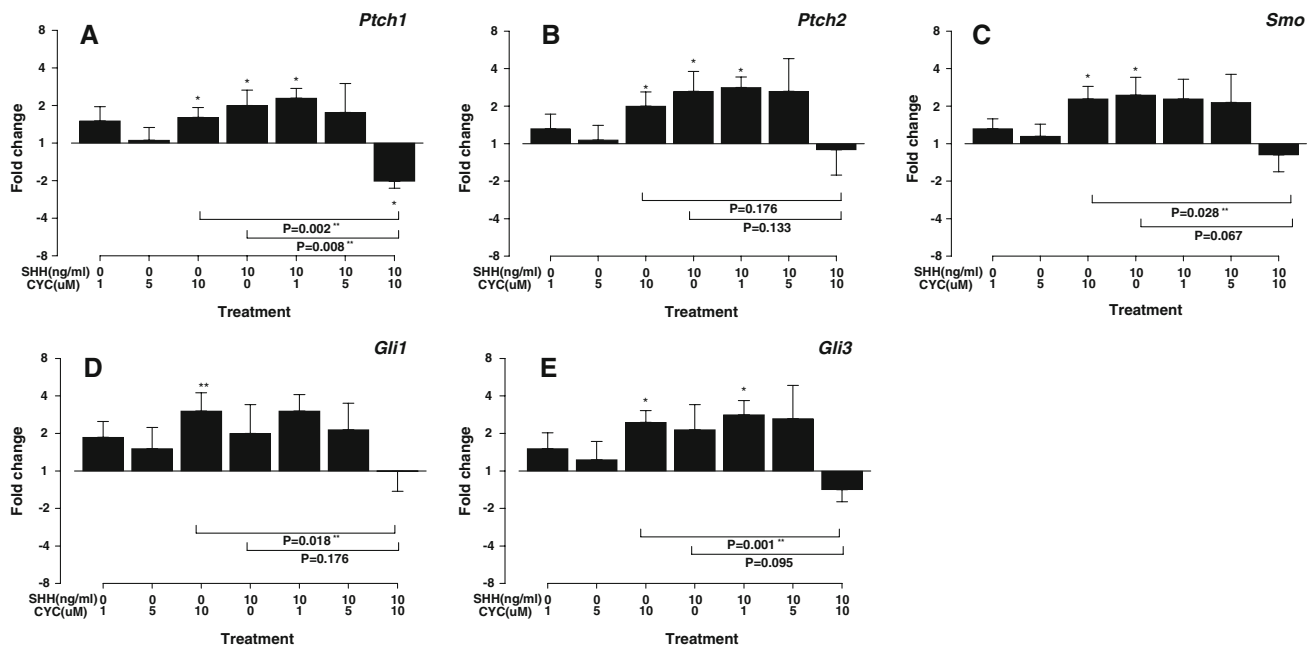


Fig. 8 Determination of the molecular response of T47D cells to simultaneous cyclopamine and SHH ligand treatment. Untreated values for each gene were normalized to “1”. Data are expressed as fold change relative to untreated, with one standard error. (a) *Ptch1* (b) *Ptch2* (c) *Smo* (d) *Gli1* and (e) *Gli3*. Statistically significant changes in gene expression relative to untreated controls are denoted

by an asterisk (*) (two-sample t-test). Similarly, significant differences between either 10 μM cyclopamine alone, or 10 ng/ml SHH-N alone and combination treatment with both 10 μM cyclopamine and 10 ng/ml SHH-N are denoted by two asterisks (**) (two-sample t-test)

growth inhibitory effects of cyclopamine at elevated dosages are independent of detectable *Smo* gene expression, and independent of specific effects on “canonical” hedgehog network gene expression.

While the cell lines used in our study overlap with those used in previous studies [9, 10], and show similar patterns of sensitivity to cyclopamine between the studies, in our hands, only two cell lines, T47D and MCF12A, expressed detectable levels of *Smo* mRNA (Fig. 1). Of these, only T47D also expressed detectable *Gli1*, a transcriptional activator that may have allowed induction of autoregulated hedgehog network genes in response to ligand treatment (Fig. 2). In contrast, MCF12A cells were not canonically responsive to ligand. Failure to detect network activation in MCF12A was consistent with the lack of detectable *Gli1* and *Gli2* expression, as well as with elevated expression of *Gli3*, and with elevated expression of both *Ptch1* and *Ptch2* relative to all other cell lines used. We did not observe a canonical molecular response to ligand treatment in any cell line in which *Smo* was undetectable. Further, we did not observe growth stimulation of any cell line in response to ligand treatment (Fig. 3).

In addition to lack of detectable *Smo* expression in cyclopamine-sensitive cell lines, we present four lines of evidence that inhibition of breast cancer cell line growth by cyclopamine may be due to activity against a second molecular target.

First, cyclopamine does not show predictable effects on hedgehog network gene expression in sensitive lines that would be consistent with inhibition of endogenous hedgehog signaling. For example, MCF7 and MDA-MB-231 cells were growth-inhibited by cyclopamine. However, these cells showed no measurable or statistically significant molecular response at the level of hedgehog network gene expression in response to cyclopamine treatment. Molecular responses of these two sensitive cell lines were similar to molecular responses of MCF10A cells, which were entirely insensitive to growth inhibition by cyclopamine.

Second, with the exception of T47D cells, which express detectable *Smo*, growth inhibition by cyclopamine cannot be rescued in competition with recombinant SHH-N ligand at doses that are capable of inducing hedgehog network gene expression approximately 2-fold in T47D cells.

Third, consistent with the possibility of a second molecular target for cyclopamine are our results evaluating the combined effect of cyclopamine and SHH-N treatment relative to each agent alone. As noted above, both 10 ng/ml SHH-N alone and 10 μM cyclopamine alone treatment induced hedgehog network gene expression to comparable levels. Given this, one might predict additive or synergistic effects on gene expression in response to treatment with both agents simultaneously. In contrast to this prediction, treatment with both agents at these doses had antagonistic effects on hedgehog network gene expression such that

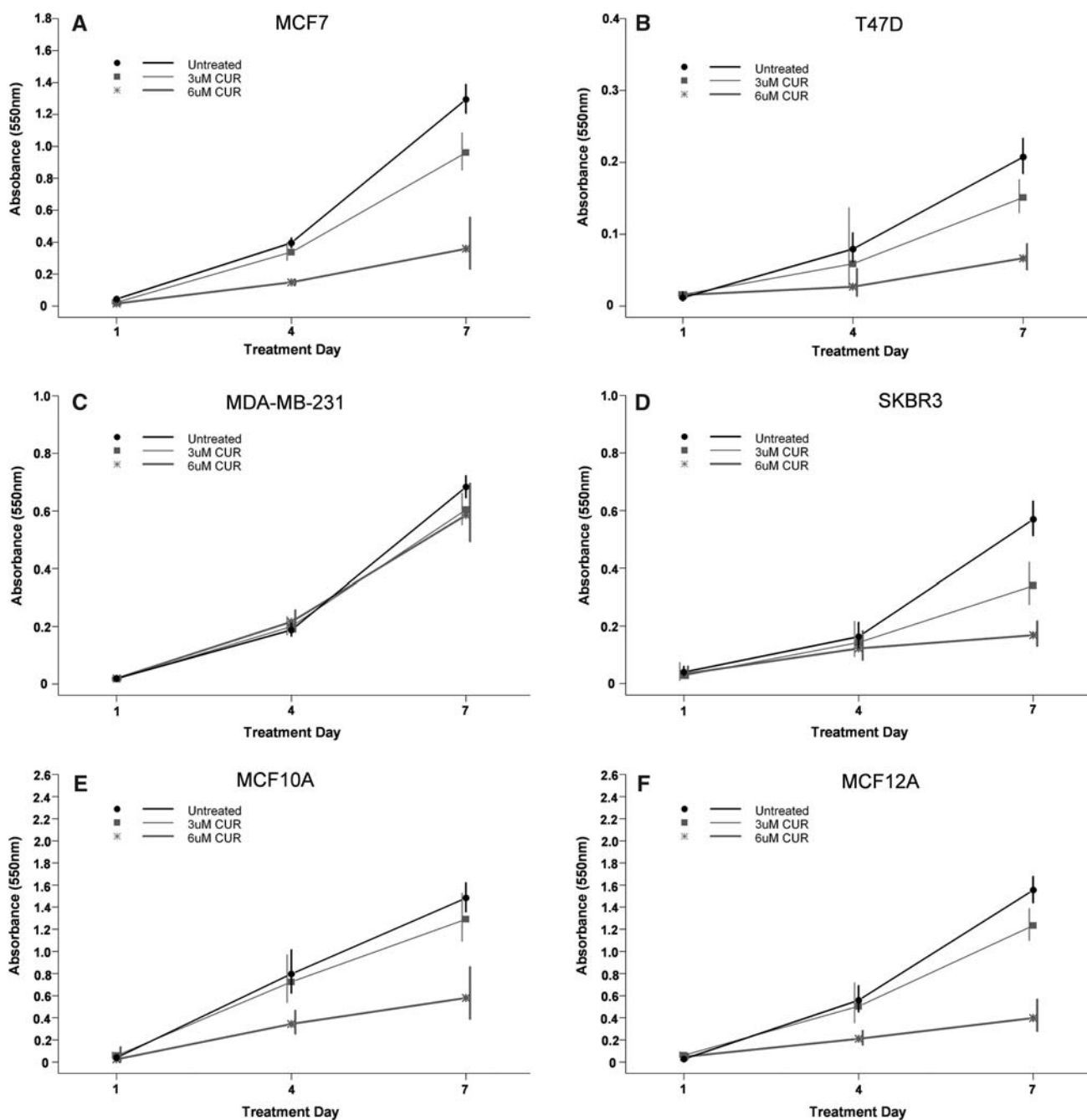


Fig. 9 Dose response curves for growth of breast cancer cell lines in response to treatment with CUR0199691. MTS assays were performed on treatment days 1, 4, and 7. Note that CUR0199691 shows

treatment resulted in levels of gene expression at, or significantly below, those of untreated cells.

Finally, and perhaps most significant, is our finding that CUR0199691, a more potent inhibitor of *Smo*-mediated hedgehog signaling than is cyclopamine, must be used at levels 100- to 1,000-fold those required for inhibition of hedgehog signaling in other models, and has a different cell type specificity than does cyclopamine. Further, treatment with CUR0199691 did not lead to significant changes in

different cell type specificity from cyclopamine. Mean absorbance for each treatment and day was plotted with 95% confidence intervals

hedgehog network gene expression in any cell line used, at any dose tested, except for an increase in *Gli1* expression in MDA-MB-231 at the 6 μM dose. Of particular note, CUR0199691 inhibited growth of two cell lines, MCF10A and MCF12A, that were entirely resistant to cyclopamine treatment even at very high doses.

Taken together, the simplest interpretation of these data is that, at lower doses (e.g. ≤ 10 μM) cyclopamine treatment may be relatively specific for inhibition of SMO-

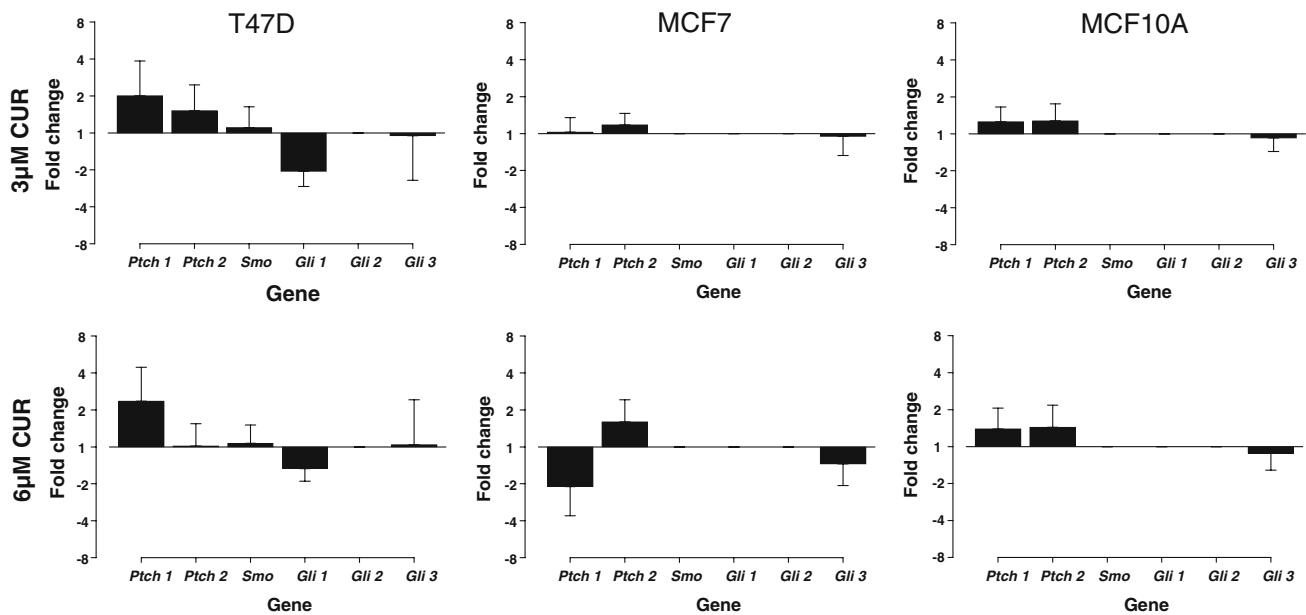


Fig. 10 Quantitative RT-PCR for hedgehog network gene expression as a function of treatment with 3 and 6 μM CUR0199691 relative to untreated cells using T47D, MCF7, and MCF10A cell lines. Untreated values for each gene were normalized to “1”. Data are

expressed as fold change relative to untreated, with one standard error. Statistically significant changes in gene expression relative to untreated cells are denoted with an asterisk (*) (two-sample t-test)

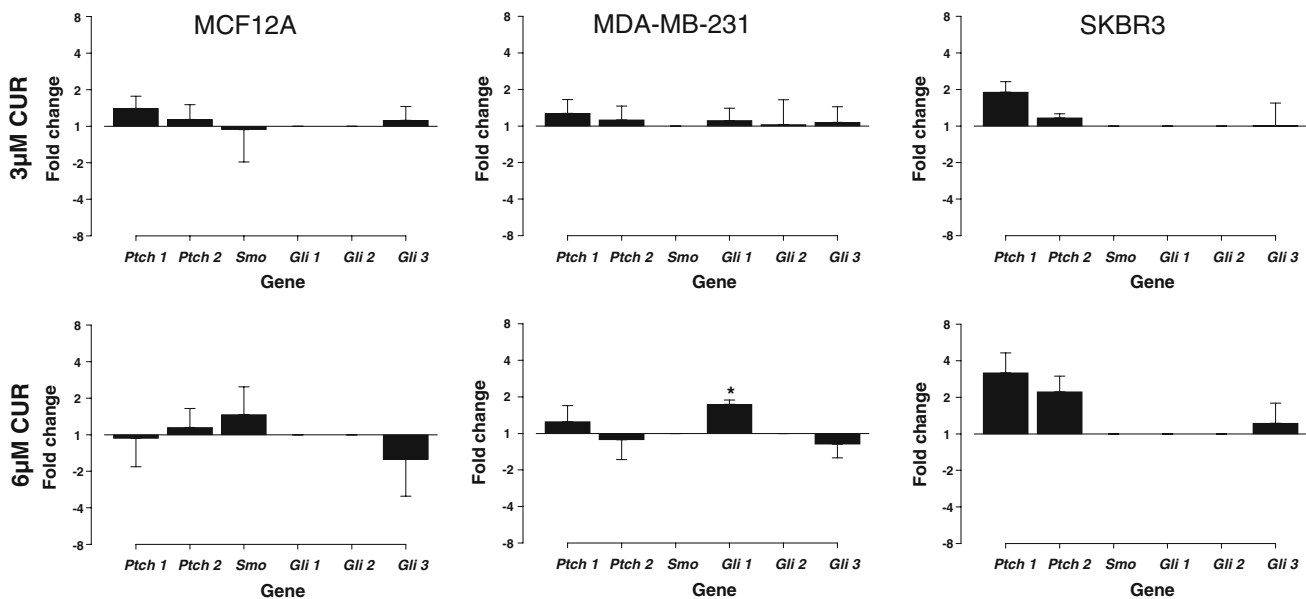


Fig. 11 Quantitative RT-PCR for hedgehog network gene expression as a function of treatment with 3 and 6 μM CUR0199691 relative to untreated cells using MCF12A, MDA-MB-231, and SKBR3 cell lines. Untreated values for each gene were normalized to “1”. Data

are expressed as fold change relative to untreated, with one standard error. Statistically significant changes in gene expression relative to untreated cells are denoted with an asterisk (*) (two-sample t-test)

mediated hedgehog signaling, but that at elevated concentrations, cyclopamine targets both hedgehog signaling and a second, as yet undefined target. Likewise, given the relatively high doses of CUR0199691 required in our study, it is likely that this compound at elevated doses also

targets both hedgehog signaling, and an undefined—but unique target. If this hypothesis is correct, cyclopamine sensitivity appears to define a critical unknown target as a factor essential for transformed, but not immortalized, non-transformed, cell growth.

Implications for use of hedgehog signaling inhibitors in breast cancer treatment

Given the recent emphasis on “target-based” or “mechanism-based” therapies for cancer treatment, the question arises whether our results should reduce enthusiasm for evaluating hedgehog signaling inhibitors in breast cancer treatment? In our opinion, they should not.

There are at least four reasons why these compounds should be pursued further. First, a primary goal in cancer research is the identification of therapeutic agents that affect cancer cell growth selectively while leaving normal cells relatively unaffected. To the extent that MCF10A and MCF12A cells approximate normal human mammary epithelial cells, and that analyses of cancer cell growth in vitro can approximate the in vivo setting, our data suggest the possibility that sensitivity to cyclopamine may correlate generally with tumorigenicity of breast cancer cells. We are aware of no other compound that shows this level of specificity for tumorigenic versus non-tumorigenic breast cancer cell line growth.

Second, in vitro evaluation of pharmacological agents can be misleading. It is possible that hedgehog signaling may play an important role in breast cancer biology in vivo, yet breast cancer cells grown in vitro may show little or no influence of hedgehog signaling on their growth. This has clearly been shown to be the case for medulloblastomas, which lose hedgehog dependency in vitro, yet are well established to be hedgehog dependent in vivo [33]. Results similar to ours have also been obtained for prostate cancer cell lines, which show little evidence of hedgehog signaling activation in vitro, yet show cyclopamine sensitivity in vivo [42].

Third, in vitro and in vivo studies indicate that aberrant *Smo* function in the epithelium can alter stem/progenitor cell fate and increase proliferation [5, 12]. However, it remains unclear whether these functions are due to autocrine or paracrine *Smo* activities [12]. Regardless of the cellular mechanism(s), interference with *Smo* function may slow or prevent tumor growth—particularly in combination with other therapies [43].

Finally, and perhaps most compelling, genetic and transplantation data in engineered mouse models suggest altered hedgehog network function in the mammary fat pad stroma can lead to profound changes in epithelial cell behavior, and lead to formation of hyperplasias strikingly similar to those of the human breast [1, 44, 45]. Thus, study of isolated breast cancer cell lines in vitro tells us nothing about whether signaling antagonists can modulate the potentially detrimental effects of increased hedgehog signaling in the mammary stroma. It is imperative that we evaluate hedgehog signaling antagonists in in vivo models in order to determine whether these compounds might be useful clinically in breast cancer treatment or prevention.

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