Combination of Cyclopamine and Tamoxifen Promotes Survival and Migration of MCF-7 Breast Cancer Cells – Interaction of Hedgehog-Gli and Estrogen Receptor Signaling Pathways

Maja Sabol¹*, Diana Trnski¹*, Zvonimir Uzarevic², Petar Ozretic¹, Vesna Musani¹, Maja Rafaj¹, Mario Cindric¹, Sonja Levanat¹*

¹. Division of Molecular Medicine, Rudjer Boskovic Institute, Zagreb, Croatia, ². Faculty of Education, Josip Juraj Strossmayer University of Osijek, Osijek, Croatia

*levanat@irb.hr

These authors contributed equally to this work.

Abstract

Hedgehog-Gli (Hh-Gli) signaling pathway is one of the new molecular targets found upregulated in breast tumors. Estrogen receptor alpha (ERα) signaling has a key role in the development of hormone-dependent breast cancer. We aimed to investigate the effects of inhibiting both pathways simultaneously on breast cancer cell survival and the potential interactions between these two signaling pathways. ER-positive MCF-7 cells show decreased viability after treatment with cyclopamine, a Hh-Gli pathway inhibitor, as well as after tamoxifen (an ERα inhibitor) treatment. Simultaneous treatment with cyclopamine and tamoxifen on the other hand, causes short-term survival of cells, and increased migration. We found upregulated Hh-Gli signaling under these conditions and protein profiling revealed increased expression of proteins involved in cell proliferation and migration. Therefore, even though Hh-Gli signaling seems to be a good potential target for breast cancer therapy, caution must be advised, especially when combining therapies. In addition, we also show a potential direct interaction between the Shh protein and ERα in MCF-7 cells. Our data suggest that the Shh protein is able to activate ERα independently of the canonical Hh-Gli signaling pathway. Therefore, this may present an additional boost for ER-positive cells that express Shh, even in the absence of estrogen.
Introduction

Breast cancer is a heterogeneous disease divided into three major subtypes with differing response to therapy: the hormone receptor-positive (with either estrogen receptor (ER) or progesterone receptor (PR) expression), the HER-2 amplified, and the triple-negative cancer (ER-negative, PR-negative and HER2-negative). For ER-positive tumors, therapy is mostly based on inhibition of estrogen synthesis or inhibition of estrogen receptor activity, for example tamoxifen is commonly used. However, many of the patients do not respond to endocrine treatment or develop acquired resistance [1].

The Hedgehog-Gli (Hh-Gli) signaling pathway is involved in embryonic development of mammary buds [2], and the pathway genes are expressed in the mammary gland during postnatal development [3]. Aberrant activation of the pathway is associated with tumorigenesis and developmental malformations. The pathway is initiated with binding of the ligand Hedgehog (Sonic, Indian or Desert Hh) to its transmembrane receptor Patched (Ptc). Ptc relieves its repression of Smoothened (Smo), causing a phosphorylation cascade and the release of transcription factor Gli from Suppressor of Fused (SuFu). Gli translocates to the nucleus, where it initiates target gene transcription. Hh-Gli pathway target genes are involved in proliferation and differentiation, cell survival, self-renewal, angiogenesis, and pathway autoregulation [4–6].

Hh-Gli signaling pathway hyperactivation has previously been detected in breast tumors [7–9]. PTCH1 gene was found downregulated due to promoter hypermethylation [10,11]. SHH promoter is frequently hypermethylated in the normal breast and this methylation is lost in breast tumors [12]. SHH is one of the signature genes associated with poor prognosis of inflammatory breast cancer [13]. Mutations in PTCH1, SMO and SHH genes have been examined in breast cancer: some studies found mutations [14,15], while others did not [16,17]. However, biallelic Pro1315Leu (C3944 T) PTCH1 polymorphism was found associated with breast cancer risk when combined with oral contraception [18]. Loss of heterozygosity of the PTCH1 gene is found in 30% of breast cancer patients [10]. The effects of cyclopamine, a Hh-Gli pathway inhibitor, on breast cancer have already been addressed in several studies. It was shown to cause growth inhibition mediated by apoptosis of some breast cancer cell lines [7,19], while cells derived from normal breast tissue are not responsive to cyclopamine [20]. The Hh-Gli signaling pathway has been implicated in tamoxifen resistance. It was shown that a small molecule SMO inhibitor GDC-0449 can improve the outcome of tamoxifen-resistant tumors. Addition of tamoxifen to GDC-0449 had additional benefits in vitro but not in vivo [21]. Recently, cyclopamine was shown to have anti-proliferative, anti-invasive and anti-estrogenic potency in human breast cancer cells by suppressing the MAPK/ERK signaling pathway. Cyclopamine decreased ERα protein levels in MCF-7 cells and the authors speculate that combining cyclopamine with anti-estrogen therapies could lower the doses and side-effects [22].
Here we show a surprising, unfavorable effect of combined inhibition of Hh-Gli signaling and ER\(\alpha\) in human ER-positive breast cancer cells and the potential underlying mechanism. In addition we also show a new, non-canonical interaction between the Hh-Gli and ER\(\alpha\) signaling pathways.

**Materials and Methods**

**Cell culture experiments**

MCF-7 (ATCC, HTB-22) and SkBr-3 (ATCC, HTB-30) breast cancer cell lines were a kind gift from Dr. Sanja Kapitanović. Both cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and were mycoplasma-free.

MTT assay: cells were plated in 96-well plates 24 hours before treatment, in quadruplicates for each tested concentration: cyclopamine 0.5–7.5 \(\mu\)M (Toronto Research Chemicals, Toronto, Ontario, Canada), tamoxifen 1–10 \(\mu\)M (Toronto Research Chemicals). Combined treatments were with either cyclopamine for 48 h followed by tamoxifen for 48 h, tamoxifen for 48 h followed by cyclopamine for 48 h, cyclopamine + tamoxifen simultaneously for 48 h, cyclopamine + tamoxifen simultaneously for 96 h. Competition experiments: compounds were added simultaneously and MTT assay was performed after 48 h.

Gene expression studies: cells were plated into 6-wells in duplicates 24 h before treatment, and treated with cyclopamine (2.5 \(\mu\)M), Shh protein (3 ng/\(\mu\)l, kind gift from Dr. Anna Kenney) and tamoxifen (1 \(\mu\)M for MCF-7, which is the LD50 dose, or 5 \(\mu\)M for SkBr-3 (LD50 was not reached for SKBr-3, therefore a higher dose was used)) for 24 h or cyclopamine + tamoxifen for 48 and 96 h.

Transfection experiments: cells were transfected with 1 \(\mu\)g of pcDNA4nlSMtGLI1 plasmid expressing the Gli1 transcription factor (kind gift from Dr. Fritz Aberger) using Lipofectamine reagent (Life Technologies, Carlsbad, California, USA). Medium was changed after 5 h and specified wells were treated with Shh protein (3 ng/\(\mu\)l); cells were collected 48 h later.

PTCH1 silencing: cells were transfected with 50 nM Silencer Select siRNA (Life Technologies, s11442) or Silencer Negative Control #1 siRNA (Life Technologies) using siPORT NeoFX (Life Technologies) transfection reagent. Medium was changed after 24 h, and cells were collected after 24 or 48 h.

**Wound healing assay**

MCF-7 cells were grown to confluence in 24-well plates and serum starved over night. The following day monolayers were wounded with a plastic 200 \(\mu\)l pipette tip and washed with medium to remove detached cells. The wounds were allowed to close in medium without any treatment or in the presence of 10 \(\mu\)M cyclopamine, 10 \(\mu\)M tamoxifen or both drugs together. Images were taken at the 0 and 26 h time points. The wounds were photographed at 10x magnification, on the Olympus CKX41 inverted microscope linked to an Olympus E330 camera.
Images were analyzed using the TScratch software, developed by the Koumoutsakos group (CSE Lab), at ETH Zürich [23]. Each time point was normalized to the 0 h image area and reported as the percent of open wound area. For the comparison of open wound areas between different treatments a one-way ANOVA with Newman-Keuls post hoc test for multiple pairwise comparisons was used. Two-tailed $p$ value less than 0.05 was considered statistically significant. Statistical analysis was performed with GraphPad Prism 6 for Windows, version 6.05 (GraphPad Software, San Diego, California, USA).

Transwell migration assay
To assay the migration of cells, $5 \times 10^4$ cells in 500 µl of serum-free medium were seeded onto 8-µm pore Transwell Inserts (Corning, Corning, NY) in the absence of any treatment or in the presence of 10 µM cyclopamine, 10 µM tamoxifen or a combination of cyclopamine and tamoxifen. The lower chambers were filled with 1 ml of complete medium. After 48 h the cells that had not migrated were wiped off the upper side of the filter using a cotton swab. Migrated cells were fixed with 4% paraformaldehyde/PBS for 10 minutes and subsequently stained with crystal violet for 1 h. Images of five independent fields per insert were taken at 20x magnification using the Olympus BX51 microscope, and the number of migrated cells was counted. For the comparison of the number of migrated cells between different treatments a one-way ANOVA with Newman-Keuls post hoc test for multiple pairwise comparisons was used.

Quantitative real-time PCR (qRT-PCR)
RNA extraction and qRT-PCR were performed as previously described [24], with primers ERα F 5′-CAGATGGTCAGTGCCTTGTTGG-3′, R 5′-CCAAGAGCAAGTTAGGAGCAAACAG-3′ [25] and RPLP0, PTCH1 and GLI1 [26,27]. Expression was normalized using RPLP0 housekeeping gene and relative fold change was calculated using the $2^{-\Delta\Delta Ct}$ formula.

Immunofluorescent staining
Immunofluorescent staining and confocal microscopy were performed as previously described [24]. The following primary antibodies diluted 1:100 were used: rabbit polyclonal anti-Hh (Santa Cruz Biotechnology, Dallas, Texas, USA, sc-9024), mouse monoclonal anti-ERα (Santa Cruz Biotechnology, sc-8002). For quantification of nuclear staining, three visual fields of magnification 60–100x were examined and cells were counted (non-treated (NT) N=79; Shh treatment N=124). Quantification of nuclear staining was obtained by determining the percent of cells showing positive ERα nuclear staining. For colocalization analysis of Shh and ERα, confocal images were examined using the Manders’ coefficient plugin of the ImageJ software (v 1.45e) for colocalization of green and red signals.
The difference in nuclear staining and co-localization between untreated samples and each treatment was tested using one-way ANOVA with Dunnett’s post hoc multiple comparisons test.

Co-Immunoprecipitation
For co-immunoprecipitation experiments Protein G Dynabeads (Life Technologies) were coated with 5 μg anti-ERα antibody per sample and cell lysates were immunoprecipitated as per manufacturer’s instructions (Invitrogen, Rev. 005). Dynabeads without bound antibody were used as negative control. Samples were eluted with 1x loading buffer and heated 10 min at 70°C before analysis on Western blot.

Western blot
Fifty μg of protein (determined by Bio-Rad Protein Assay; Bio-Rad, Hercules, California, USA) was loaded on SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and blocked in 5% milk. Primary antibodies (diluted 1:250) for Shh and ERα were the same as for the immunofluorescence experiment, additionally goat polyclonal anti-Ptch1 (Santa Cruz Biotechnology, sc-6147) and rabbit polyclonal anti-Gli1 (Santa Cruz Biotechnology, sc-20687) were used. Actin (Santa Cruz Biotechnology, sc-1616, goat polyclonal, diluted 1:500) was used as loading control. After washing, membranes were incubated with the appropriate secondary HRP-conjugated antibody (Santa Cruz Biotechnology). Proteins were visualized using Super Signal West Pico and Femto reagents (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Proteomic profiling by 2D-gel electrophoresis and mass spectrometry
Sample preparation
Cells were seeded in four 10 cm dishes for each treatment. After 24 h cells were treated with a combination of 5 μM cyclopamine and 10 μM tamoxifen in culture medium without serum for 48 h. The cells were then harvested at 4000 g (Tehtnica, Centric 400, Železniki, Slovenia) for 6 min, washed five times in 10 mM Tris (hydroxymethyl) aminomethane (Tris)-sorbitol buffer, pH 7 and lysed with TissueRuptor (Qiagen, Venlo, Netherlands). The DNA and RNA were removed after treatment with DNase I and RNase A. The reconstituted proteins were precipitated overnight at −20°C with ice-cold acetone and centrifuged for 20 min at 5000 g [29]. The proteins were resuspended in rehydration solution for isoelectric focusing (IEF) containing 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate hydrate (CHAPS) and 1% dithiothreitol (DTT) (w/v). Protein concentration in solution was estimated with Bradford protein assay.
Two-dimensional electrophoresis

Immobilized pH gradient strips (IPG; 17 cm, non-linear, pH 3–10) were rehydrated for 14 h with 350 mL of rehydration solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT (w/v) and 1.5 mg/mL of total protein. The IEF was carried out with a Protean IEF Cell (Bio-Rad) with a low initial voltage and an applied voltage gradient up to 7000 V. The total V × t product applied was 90 000 Vh for each strip at 20˚C. The strips were equilibrated in equilibration buffer containing 20 mM DTT, 50 mM Tris adjusted to pH 6.8, 6 M urea, 2% sodium dodecyl sulfate (SDS) (w/v), 30% glycerol (v/v) and 0.01% bromophenol blue (BPB) (w/v) on a tilt table for 15 min. The solution was discarded and the same equilibration buffer solution without the addition of DTT and with the addition of 25 mM iodoacetamide was used for a 15 min protein alkylation reaction. The strips were placed on a 1 mm thick 12% polyacrylamide gel and sealed with 0.1% (w/v) agarose in SDS-electrophoresis buffer containing 0.01% (w/v) BPB. In the second dimension, the electrophoresis was run for 1 h at 15 mA per gel and then at 20 mA for 600 Vh. The electrophoresis was terminated after 30 mA per gel until the BPB reached the bottom of the gel. Tris-glycine running buffer containing 25 mM Tris, 190 mM glycine and 0.1% (w/v) SDS was used in the second dimension. Obtained gels were stained with Coomassie brilliant blue (CBB) G-250 stain [30].

Differential display analysis

Differential display analysis of the gel data sets was undertaken by comparing images of control gel (non-treated cell cultures) with the gel of treated cells (combination of cyclopamine and tamoxifen). Densitometry analysis was performed with image analysis software (Discovery Series PDQuest 2-DE analysis software package version 7.4.0.) integrated with a VersaDoc 4000 Imaging System (Bio-Rad). Master gels were used to obtain the differences between protein profiles of non-treated and treated cell cultures.

In-gel digestion

Differentially displayed protein spots were excised from 2-DE gels into small pieces and subjected to in-gel digestion with trypsin according to Shevchenko et al [31].

Data analysis and protein identification

Samples were mixed with α-cyano-4-hydroxycinnamic acid 1:5, v/v (5 mg/mL; Fluka, Switzerland) and spotted onto a metal plate. MS acquisition was performed with a 4800 Plus MALDI TOF/TOF analyzer (Applied Biosystems, Carlsbad, California, USA) equipped with a 200 Hz, 355 nm Nd:YAG laser. Ions were analyzed in reflectron mode using positive polarity. The instrument parameters were set using the 4000 Series Explorer software (version 3.5.3, Applied Biosystems). Mass spectra were obtained by averaging 1000 laser shots covering a mass range of m/z 900 to 4000. MS/MS of the 10 most intense precursor signals
from MS spectra was achieved by 1 keV collision energy in positive ion mode with air as a collision gas and by averaging 1600 laser shots.

Data were analyzed using ProteinPilot (ProteinPilot™ Software 4.5., 2012 AB SCIEX) [32] for searching against the NCBI database using the Homo sapiens taxonomy. The search parameters allowed for two missed cleavage, trypsin digestion with a peptide tolerance = 0.3 Da and MS/MS tolerance = 0.5 Da. Only significant scores (greater than 39, p < 0.05) for the peptides defined by a Mascot probability analysis were considered to be confidently identified peptides/proteins.

Results

MCF-7 and SkBr-3 cells are responsive to cyclopamine and tamoxifen treatment – combination shows unusual adverse effects

Both the ER-positive MCF-7 and the ER-negative SkBr-3 show expression of Hh-Gli pathway components. The major difference between the two cell lines was the expression of Shh and ERα, while the MCF-7 cell line expressed Shh and ERα both on gene and protein (Shh-N, 19 kDa) level, SkBr-3 cells showed low levels of SHH and ERα gene expression and no expression at protein level (Fig. 1A, B). SkBr-3 cells also showed no expression of GLI1 (Fig. 1A).

MCF-7 cells were responsive to both Hh-Gli signaling downregulation with cyclopamine, and ERα inhibition with tamoxifen, which both decreased MCF-7 cell proliferation. Both treatments had a significantly weaker effect on the ER-negative SkBr-3 cell line (Fig. 2A–D). To determine the effects of a combined treatment on cell proliferation, cells were treated with cyclopamine and tamoxifen in four different combinations: cyclopamine for 48 h followed by tamoxifen for 48 h, tamoxifen for 48 h followed by cyclopamine for 48 h, cyclopamine + tamoxifen simultaneously for 48 h and 96 h (Fig. 2E, F). In most cases, the combined effect was very similar to the effect of tamoxifen alone. However, a short-term combined treatment did not cause significantly decreased proliferation in MCF-7 cells (Fig. 2E).

We tested the possible competition of cyclopamine and tamoxifen in both cell lines: cells were treated with a constant concentration of one compound, combined with a range of increasing concentrations of the second compound. For SkBr-3 cell line, there was no significant difference in compound activity (data not shown). In the MCF-7 cell line, however, increasing concentrations of the second compound increased short-term cell survival; regardless of the order of administration (Fig. 3). This suggests that even though cyclopamine and tamoxifen alone show inhibitory effects on MCF-7 cells, when administered together they counter each other’s effects.
Combined cyclopamine and tamoxifen treatment alters Hh-Gli signaling pathway activity in MCF-7 cells and promotes cell migration

Prior to investigating the combined effect of cyclopamine and tamoxifen, we first tested the effect of cyclopamine and tamoxifen alone on the Hh-Gli signaling pathway. Both cell lines showed a similar response when treated with cyclopamine. 24 h after treatment with cyclopamine, PTCH1 and GLI1 expression was downregulated in the MCF-7 cell line and PTCH1 was downregulated in SkBr-3, suggesting pathway inhibition (Fig. 4A). Tamoxifen treatment upregulated PTCH1 and GLI1 expression in MCF-7 cells, while PTCH1 levels remained unchanged in the SkBr-3 cell line (Fig. 4B).

Although some pathway components are expressed, the pathway shows a low level of activity in SkBr-3 cells, but with downregulation possibility with cyclopamine, which may be carried out through other pathway effectors such as Gli2 or Gli3 that were not tested in this study.

Combined treatment with cyclopamine and tamoxifen showed a different effect on ER-positive and ER-negative cell line. ER-positive MCF-7 cell line showed increased Hh-Gli signaling after short-term treatment. Even though the level of PTCH1 mRNA was still elevated after long-term treatment, a decreasing tendency was visible compared with short-term treatment. This is confirmed by the level of PtcH1 protein, which was decreased 40% after long-term combined treatment compared with non-treated cells. ERα protein level showed no change after shorter treatment but declined after longer treatment (Fig. 4C). SkBr-3, however, showed generally downregulated Hh-Gli signaling after combined treatment regardless of treatment duration (Fig. 4D).

Wound induced migration assay was performed to test whether the combination of cyclopamine and tamoxifen has an effect on the ability of MCF-7 cells to migrate, in addition to the effects on Hh-Gli signaling and cell...
proliferation. Cyclopamine or tamoxifen alone had no effect on the wound closing rate, compared with the wound closing in the absence of any treatment. On the other hand, combined treatment with cyclopamine and tamoxifen accelerated the wound healing process compared with non-treated conditions and with cyclopamine or tamoxifen alone (Fig. 4E, F). To confirm the obtained results a transwell migration assay was performed. This assay confirmed no effect of either cyclopamine or tamoxifen alone on the migration rate when compared with the non-treated cell migration rate. It also confirmed a higher migration capacity of MCF-7 cells treated with a combination of cyclopamine and tamoxifen compared
with non-treated cells or cells treated with cyclopamine or tamoxifen alone (Fig. 4G, H). The increase in the migration capacity was even higher when analyzed with the transwell migration assay in comparison with the wound healing assay.

Proteomic profiling of cells treated with cyclopamine and tamoxifen versus non-treated cells

Differential protein expression analysis was conducted to identify the profile of expressed proteins in cells treated with a combination of cyclopamine and tamoxifen. These differentially expressed proteins may explain the effects of the combined treatment with cyclopamine and tamoxifen on cell proliferation and migration. The identified proteins are listed in Table 1. Images of the obtained 2-D gels are shown in S1 Figure. As opposed to cells treated with a combination of drugs, non-treated cells mostly show expression of proteins involved in response to topologically incorrect and unfolded proteins; carbohydrate and amino acid metabolism, gene transcription, RNA processing and translation. Interestingly, the heat shock protein 27 (HSP27) is expressed in both non-treated cells and those treated with a combination of cyclopamine and tamoxifen. However, the protein is shifted in the 2-D gel of treated cells compared with its localization in the 2-D gel of non-treated cells, which could indicate a posttranslational modification after treatment. Additionally, the GRP78 precursor protein, which is a known survival factor [33] that can mediate signaling pathways that lead to proliferation and invasion [33, 34] was expressed only in treated cells. Also, two proteins that can be linked with upregulation of proliferation and migration showed an increase in expression in treated cells, namely prohibitin and keratin 8 [35, 36]. Together these results indicate that certain proteins involved in tumor cell survival and migration are upregulated or possibly activated.
Fig. 4. Effects of cyclopamine (A) and tamoxifen (B) on Hh-Gli pathway gene expression in MCF-7 and SkBr-3 cells. The Hh-Gli pathway is upregulated after short-term combined treatment in MCF-7, but the effect is negated after longer treatment. On the Western blot image, band quantification relative to actin and non-treated cells is denoted below the bands. (C). The effect of combined treatment on SkBr-3 cell line is weak (D). Gene expression levels are shown on graph as relative fold change relative to non-treated conditions with reference value 1 pointed out with emboldened bar. Only combined cyclopamine and tamoxifen treatment induces migration in MCF-7 cells. Representative images of the wound healing assay at 0 and 26 h (after processing with TScratch software [23]) are shown for non-treated conditions (NT; N = 16), cyclopamine treatment (CYC; N = 16), tamoxifen treatment (TAM; N = 14) and combined treatment with cyclopamine and tamoxifen (C+T; N = 12) (E). Quantitative analysis of the percentage of open wound areas is shown on the graph,
Shh regulates ERα expression in MCF-7, but not SkBr-3 cell line

Since inhibition of ERα with tamoxifen affected Hh-Gli signaling we wanted to establish whether there is cross-talk between these two pathways. Therefore, both cell lines were treated with Shh protein. MCF-7 cells responded to stimulation with exogenous Shh protein by Hh-Gli pathway activation (Fig. 5A, C) whereas the ER-negative cell line did not respond to Shh stimulation (Fig. 5B). Interestingly, short-term Shh treatment also had an effect on ERα expression in ER-positive cell line, which was increased (Fig. 5D, F), but this effect was relatively quickly negated 48 h post-treatment (Fig. 5D). In the SkBr-3 cell line there was no upregulation of ERα in response to Shh protein, but rather a slight down-regulation (Fig. 5E).

To check whether the effect of Shh on ERα is mediated via the canonical Hh-Gli signal transduction, cells were transfected with GLI1. After transfection and additional Shh stimulation, Gli1 and Ptch1 gene and protein expressions were elevated in MCF-7 cells (Fig. 6C, S2 Figure), whereas ERα was upregulated in MCF-7 cell line only after exogenous Shh stimulation (Fig. 6A). On the protein level ERα expression decreased after GLI1 transfection, but an increase was visible after Shh addition, compared with only transfected cells (Fig. 6C). This suggests that ERα regulation is not mediated transcriptionally via Gli1 transcription factor, but rather directly by Shh protein.

Even though the transfection was successful in SkBr-3 cells, shown by upregulation of GLI1 and PTCH1 expression (S2 Figure), it had no effect on ERα gene expression which was expected since there is only a low basal level of ERα mRNA expression and no ERα protein production in these cells (Fig. 6B).

To confirm a direct impact of Shh protein on ERα we silenced PTCH1, the primary Shh receptor, which would cause an increase in free, unbound Shh protein that could in turn interact with ERα and increase its activity. The effect was induction of ERα expression in MCF-7 cells, suggesting Shh protein has a direct effect on ERα. (Fig. 6D, F) For SkBr-3 cell line, sufficient knockdown of PTCH1 was achieved 48 h post-transfection (Fig. 6E) and the effect on ERα was downregulation of gene expression (Fig. 6G).

Shh protein interacts with ERα

To verify whether Shh has a direct effect on ERα, cells were treated with Shh protein, for 48 h and localization of Shh and ERα was visualized. Non-treated cells showed Shh staining in a granular pattern in the cytoplasm, mostly surrounding the nucleus, while ERα was scattered in the cytoplasm and stronger in the nuclei. Shh treatment caused an interesting effect: co-localization of Shh
Table 1. Differentially expressed proteins in MCF-7 cells treated with cyclopamine and tamoxifen compared with non-treated control cells.

<table>
<thead>
<tr>
<th>No</th>
<th>Protein Description</th>
<th>GI Accession</th>
<th>Score</th>
<th>General Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Heat shock protein 90-alpha</td>
<td>gi</td>
<td>32488</td>
<td>142</td>
</tr>
<tr>
<td>2</td>
<td>Ezrin</td>
<td>gi</td>
<td>11276938</td>
<td>110</td>
</tr>
<tr>
<td>3</td>
<td>KHSRP protein</td>
<td>gi</td>
<td>54648253</td>
<td>145</td>
</tr>
<tr>
<td>4</td>
<td>Heat shock protein 75</td>
<td>gi</td>
<td>2865486</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>TATA-binding protein-associated factor 2N isoform 2</td>
<td>gi</td>
<td>4507353</td>
<td>52</td>
</tr>
<tr>
<td>6</td>
<td>Alpha-tubulin</td>
<td>gi</td>
<td>340021</td>
<td>232</td>
</tr>
<tr>
<td>7</td>
<td>Pyrroline-5-carboxylate dehydrogenase</td>
<td>gi</td>
<td>1353248</td>
<td>81</td>
</tr>
<tr>
<td>8</td>
<td>UDP-glucose 6-dehydrogenase isoform 1</td>
<td>gi</td>
<td>4507813</td>
<td>72</td>
</tr>
<tr>
<td>9</td>
<td>Translation initiation factor 4A–III</td>
<td>gi</td>
<td>496902</td>
<td>144</td>
</tr>
<tr>
<td>10</td>
<td>Glutamate dehydrogenase 1, mitochondrial precursor</td>
<td>gi</td>
<td>4885281</td>
<td>64</td>
</tr>
<tr>
<td>11</td>
<td>Alpha-enolase isoform 1</td>
<td>gi</td>
<td>4503571</td>
<td>119</td>
</tr>
<tr>
<td>12</td>
<td>Laminin-binding protein</td>
<td>gi</td>
<td>34234</td>
<td>170</td>
</tr>
<tr>
<td>13</td>
<td>Keratin 10</td>
<td>gi</td>
<td>28317</td>
<td>51</td>
</tr>
<tr>
<td>14</td>
<td>Heat shock protein 27</td>
<td>gi</td>
<td>35182</td>
<td>124</td>
</tr>
</tbody>
</table>

Proteins with ≥2 times lower expression in MCF-7 cells treated with cyclopamine + tamoxifen compared with control cells

<table>
<thead>
<tr>
<th>No</th>
<th>Protein Description</th>
<th>GI Accession</th>
<th>Score</th>
<th>General Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>far upstream element-binding protein 1</td>
<td>gi</td>
<td>17402900</td>
<td>172</td>
</tr>
<tr>
<td>19</td>
<td>far upstream element-binding protein 1</td>
<td>gi</td>
<td>17402900</td>
<td>172</td>
</tr>
<tr>
<td>20</td>
<td>Heterogeneous nuclear ribonucleoprotein H</td>
<td>gi</td>
<td>5031753</td>
<td>116</td>
</tr>
<tr>
<td>21</td>
<td>Elongation factor 1 alpha</td>
<td>gi</td>
<td>31092</td>
<td>40</td>
</tr>
<tr>
<td>22</td>
<td>Tu translation elongation factor, mitochondrial, isoform CRA_b</td>
<td>gi</td>
<td>119572383</td>
<td>148</td>
</tr>
<tr>
<td>23</td>
<td>C protein</td>
<td>gi</td>
<td>306875</td>
<td>97</td>
</tr>
<tr>
<td>26</td>
<td>Triosephosphate isomerase</td>
<td>gi</td>
<td>136066</td>
<td>75</td>
</tr>
</tbody>
</table>
2-D gel of MCF-7 cells treated with cyclopamine + tamoxifen

<table>
<thead>
<tr>
<th>No</th>
<th>Protein Description</th>
<th>GI Accession</th>
<th>Score</th>
<th>General Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>GRP78 precursor, partial</td>
<td>gi</td>
<td>386758</td>
<td>133</td>
</tr>
<tr>
<td>29</td>
<td>Heat shock protein 27</td>
<td>gi</td>
<td>662841</td>
<td>91</td>
</tr>
</tbody>
</table>

Proteins with ≥2 times higher expression in MCF-7 cells treated with cyclopamine + tamoxifen compared with control cells

<table>
<thead>
<tr>
<th>No</th>
<th>Protein Description</th>
<th>GI Accession</th>
<th>Score</th>
<th>General Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>Keratin 8, isoform CRA_a</td>
<td>gi</td>
<td>119617057</td>
<td>76</td>
</tr>
<tr>
<td>32</td>
<td>Prohibitin isoform 1</td>
<td>gi</td>
<td>4505773</td>
<td>308</td>
</tr>
</tbody>
</table>

General Functions are obtained from the UniProt and NCBI Gene databases. Protein numbers correspond to the numbers marked on the 2-D gels (Figure S1). Numbers in the table correspond to spot numbers denoted on the 2-D gel images; missing numbers in the table are unidentified proteins or proteins with score less than 39.

doi:10.1371/journal.pone.0114510.t001

Fig. 5. Effect of stimulation with Shh protein on pathway activity in MCF-7 (A,C) and SkBr-3 cells (B). Gene expression levels are shown on graph as relative fold change relative to non-treated conditions with reference value 1 pointed out with emboldened bar. Relative gene expression of ERα after treatment with Shh protein (D,E). Non-treated cells (NT) have a relative value 1. ERα protein expression in MCF-7 cells increases after treatment with Shh protein for 48 h (F) Protein bands were quantified and normalized relative to actin and non-treated conditions and the relative values are denoted below each band.

doi:10.1371/journal.pone.0114510.g005
and ERα in the cytoplasm of the cells (Fig. 7A). There was very little co-localization of ERα and Shh in untreated cells, but after 48 h-treatment with Shh protein there is significantly less nuclear staining of ERα (P<0.0003) and ERα and Shh co-localized in the cytoplasm (P<0.0001) (Fig. 7B). This suggests that Shh acts directly on ERα, modifying its activity. Co-immunoprecipitation results however, indicate an interaction of Shh and ERα proteins in general, regardless of treatment with exogenous Shh protein (Fig. 7C). This is not unusual as the MCF-7 cells produce high amounts of Shh protein. These results undoubtedly show an interaction between Shh and ERα proteins, which is the first mention of direct interaction between these two proteins. However, adding exogenous Shh protein did not increase this interaction, as would be expected from the immunofluorescence data. It is possible that, since the MCF-7 cells already produce high amounts of Shh protein, addition of exogenous protein has no influence on the interaction rate. However, the fact that there is an obvious interaction between these two proteins is a new and intriguing finding that needs to be investigated further as it opens new possibilities in the aspect of Hh-Gli signaling research in ER-positive breast cancer.
Fig. 7. Immunofluorescent staining of MCF-7 cell line in non-treated cells (NT) and treated with Shh protein detected by confocal microscopy. ERα is stained green (column 1), Shh is stained red (column 2), nuclei are stained blue with DAPI (column 3), and the last column shows the overlay of signals. Yellow
Discussion

The role of Hh-Gli signaling in breast cancer is still unclear, especially regarding their association with steroid receptor signaling. To date the findings of Hh-Gli component expression in breast cancer cell lines is contradictory, particularly for Shh and Gli1. We found expression of Gli1 and Shh in the ER-positive cell line (MCF-7), but Ramaswamy et al. on the other hand found no expression of Shh in MCF-7 cells [21]. This inconsistency may be due to the fact that the authors looked only at the expression of unprocessed Shh protein (45 kDa). This is supported by the expression of SHH at the mRNA level which they did find. Two other studies, on the other hand, did find Shh expression in MCF-7 cells [7, 37]. Also, some studies show high expression of GLI1 in ER-negative cell lines, including SkBr-3 [20, 38], but in our hands GLI1 expression was not detectable in SkBr-3 cells. Recently a study showed a positive correlation between ERα and GLI1 expression [39], supporting lower levels of GLI1 in the ER-negative cell line. Even though these authors did find very low GLI1 expression in SkBr-3 it was much lower than in MCF-7. Given the lower levels of GLI1 in MCF-7 cells that we detected it is not surprising it was undetectable in SkBr-3.

Cyclopamine has been tested together with gefitinib in prostate cancer cell lines, where the combined treatment induced a supra-additive inhibitory growth effect on serum-free and serum-stimulated cell lines. This effect is established through cell cycle arrest in G1 phase and increased apoptosis. Cyclopamine and gefitinib-treated cells showed a decreased ability for invasion, and this effect was amplified in combined treatment [40]. In other studies on prostate cancer cells cyclopamine used in combination with ErbB inhibitors gefitinib or lapatinib showed a synergistic effect [41, 42] and combination of docetaxel+cyclopamine+gefitinib induced more intensive cell death compared to either treatment alone [43]. In cholangiocarcinoma treatment with cyclopamine and MEK inhibitor U0126 showed an additive effect, especially in cells with KRAS mutation [44].

Our results regarding the effect of cyclopamine on breast cancer cells are in agreement with previous studies that have shown that cyclopamine inhibits human breast cancer cell growth by increased apoptosis [19]. In a study by Che et al. [22] cyclopamine was reported to have anti-proliferative, anti-invasive and anti-estrogenic potency in human breast cancer. This is similar to our findings which also showed the anti-estrogenic effect of cyclopamine, ERα gene expression was downregulated after cyclopamine treatment.

In the ER-positive breast cancer cell line, however, combined treatment with cyclopamine and tamoxifen increased cell viability after short-term treatment, but it was not seen in ER-negative cells. This effect was dose-dependent, and
competition experiments have shown that higher concentrations of both compounds are required for the survival effect. Short-term combined treatment of MCF-7 cells upregulated the Hh-Gli signaling pathway and promoted cell migration (Figs. 2–4).

To elucidate the effect of the combination of these two drugs on the profile of expressed proteins we performed proteomic profiling of cells treated with a combination of cyclopamine and tamoxifen as well as control non-treated cells. This analysis revealed that a small but unique set of proteins is upregulated upon combination treatment in comparison with non-treated cells. All of them have been linked to cell proliferation and migration (Table 1). GRP78, a known survival factor, has been known to mediate signaling pathways that lead to proliferation an migration [33, 34]. Prohibitin was initially shown to block cell proliferation [45], but this ability was attributed to its 3’ untranslated region [46]. However, there is emerging evidence that prohibitin as a protein is required for cell proliferation and adhesion [47]. This protein is also known for activating the Raf-MEK-ERK signaling pathway and inducing cell migration [36, 48]. Another protein found to be upregulated after treatment with cyclopamine and tamoxifen is keratin 8. The data on the role of keratin 8 in cancer are inconsistent. Some studies show that keratin 8 overexpression correlates with lower tumorigenicity, invasiveness and motility [49], while others found it to be correlated with poor prognosis, invasiveness and cell migration [35, 50, 51]. HSP27, which is expressed under stressful conditions, is found both in treated cells and non-treated cells, but the protein was shifted in relation to the protein in non-treated cells suggesting it was modified. It has been found that the phosphorylated form of this protein participates in stress resistance and act as a negative regulator of apoptosis and a positive regulator of proliferation and migration [52–55]. This suggests that a combination of these drugs potentially enhances the migration ability of these cells, which is consistent with the results obtained by the wound healing and transwell migration assays, showing that cells treated with the combination of drugs have a higher migration capacity than the non-treated ones. Whether this effect is related to the upregulation of the Hh-Gli signaling pathway remains to be investigated. It should be looked into whether the Hh-Gli signaling pathway can directly or indirectly affect the expression of these proteins.

Apart from Hh-Gli pathway being regulated by compounds affecting ERα (tamoxifen), the communication works also in the other direction, from Hh-Gli signaling to ERα. The link between ERα and Hh-Gli signaling pathways has been addressed in previous studies. It was shown that upregulation of ERα by E2 also upregulated Shh which canonically activated Hh-Gli signaling and Gli1 expression in human breast cancer cells [37]. The same link was observed in ERα positive gastric cancer [56]. In both studies the vice versa link was not observed. We on the other hand, show a potential mechanism of ERα regulation through Hh-Gli signaling. Although there may be a transcriptional link between Hh-Gli and estrogen signaling via FoxM1 [25, 57], this does not seem to be the case here. Transfection of GLI1 does not automatically induce transcription of ERα, like it does of PTCH1; suggesting ERα expression is not regulated transcriptionally via
Gli1. Only after exogenous addition of Shh protein there is an induction in ERα, regardless of GLI1 levels. Our co-immunoprecipitation assay confirmed a direct link between Shh and ERα proteins (Figs. 5–7). It is possible that the cholesterol modification of the Shh protein plays a role in this interaction since cholesterol is the precursor molecule for steroid hormones, but this remains to be analyzed. This interaction may be the cause of upregulation of ERα activity and consequently upregulation of ERα gene and protein expression. Silencing of PTCH1 leads to a reduced number of receptor molecules on the membrane, allowing increased binding of endogenous Shh to the ERα, which leads to upregulation of ERα expression (Fig. 6), since ERα autoregulates its own expression [58].

The mechanism which is responsible for the increased viability of ER-positive cell line after combined treatment with cyclopamine and tamoxifen, in comparison with either treatment alone, is not clear. We show that the Hh-Gli signaling is upregulated and proteins involved in proliferation and migration enhancement are expressed, but the link between them and the Hh-Gli signaling remains to be elucidated. Although Hh-Gli signaling seems to be a good potential target for breast cancer therapy, caution must be advised, especially when combining therapies. We have demonstrated that combined treatment of cyclopamine and tamoxifen may induce an opposite effect, providing cells with short-term survival and increased ability to migrate, which may be deleterious for the patient. On the other hand, we show a potential direct link between Shh and ERα proteins. According to our results Shh can bind ERα and activate it. This might be a mechanism that enhances survival of breast cancer cells with expression of Shh, even in estrogen deficient conditions.

Supporting Information

S1 Figure. 2-D gels of non-treated control MCF-7 cells (A) and MCF-7 cells treated with cyclopamine and tamoxifen (B). 2-D gel of MCF-7 cells treated with a combination of cyclopamine and tamoxifen with indicated spots that have ≥2 times higher expression compared with control cells (C). 2-D gel of MCF-7 cells treated with a combination of cyclopamine and tamoxifen with indicated spots that have ≥2 times lower expression compared with control cells (D). Indicated spots were used for further MS analysis. Results are shown in Table 1.
doi:10.1371/journal.pone.0114510.s001 (TIF)

S2 Figure. GLI1 and PTCH1 gene expression levels after transfection with GLI1 plasmid in ER-positive MCF-7 cells (A, C) and ER-negative SkBr-3 cells (B, D).
doi:10.1371/journal.pone.0114510.s002 (TIF)

Acknowledgments

The authors wish to thank Lucija Horvat, B.Sc. for help with confocal microscopy and Mirela Levacic Cvok, B.Sc. for all the help with the laboratory work. We thank
Dr. Marijeta Kralj for use of the Olympus CKX41 microscope linked to the Olympus E330 camera and Dr. Mirko Hadzija and Dr. Marijana Popovic Hadzija for use of the Olympus BX51 microscope.

**Author Contributions**
Conceived and designed the experiments: MS SL. Performed the experiments: MS DT ZU PO VM MR. Analyzed the data: MS DT PO VM MC SL. Contributed reagents/materials/analysis tools: MC. Contributed to the writing of the manuscript: MS DT SL.

**References**


