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# Hispolon inhibits the growth of estrogen receptor positive human breast cancer cells through modulation of estrogen receptor alpha

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ABSTRACT

Human estrogen receptor α (ERα) is a nuclear transcription factor that is a major therapeutic target in breast cancer. The transcriptional activity of ERα is regulated by certain estrogen-receptor modulators. Hispolon, isolated from *Phellinus linteus*, a traditional medicinal mushroom called Sanghwang in Korea, has been used to treat various pathologies, such as inflammation, gastroenteric disorders, lymphatic diseases, and cancers. In this latter context, Hispolon has been reported to exhibit therapeutic efficacy against various cancer cells, including melanoma, leukemia, hepatocarcinoma, bladder cancer, and gastric cancer cells. However, ERα regulation by Hispolon has not been reported. In this study, we investigated the effects of Hispolon on the growth of breast cancer cells. We found that Hispolon decreased expression of ERα at both mRNA and the protein levels in MCF7 and T47D human breast cancer cells. Luciferase reporter assays showed that Hispolon decreased the transcriptional activity of ERα. Hispolon treatment also inhibited expression of the ERα target gene pS2. We propose that Hispolon, an anticancer drug extracted from natural sources, inhibits cell growth through modulation of ERα in estrogen-positive breast cancer cells and is a candidate for use in human breast cancer chemotherapy.


## 1. Introduction

Breast cancer is the most common cancer in women, affecting millions worldwide [1]. The pathogenesis of breast cancer has been linked to hormone stimulation of estrogen receptor alpha (ERα) [2]. ERα, a transcription factor that regulates a large number of genes in many different target tissues, is critical in the development and progression of breast cancer [3]. The classic mechanism of ER signaling is through direct action of ligand-bound ER. Ligand binding induces a conformational change in the ERα that promotes its disassociation from the chaperone protein HSP90 [4]. This is followed by ERα dimerization and binding to estrogen-response elements (EREs) in estrogen-responsive genes, such as pS2. Increased transcription of these genes enhances mammary epithelial cell proliferation and tumorigenesis [5]. Because of the breast cancer-promoting role of ERα signaling, ERα has been the focus of the development of anti-estrogen therapy in ERα-positive breast cancer cells. Anticancer drugs, such as tamoxifen, that have both antagonist and agonist activities exert their anti-tumor activity by blocking ERα activity and by recruiting co-repressors to the estrogen-ERα complex [5,6]. However, their partial agonist activity causes some harmful effects, such as an increased risk of other cancers in women with prolonged treatment [7]. This has led to a search for new breast cancer treatments.

*Phellinus linteus*, a traditional medicinal mushroom called Sanghwang in Korea, has been used to treat various pathological conditions, including inflammation, gastroenteric disorders, cancers, and lymphatic diseases [8–11]. Hispolon [6-(3,4-dihydroxyphenyl)-4-hydroxyhexa-3,5-dien-2-one], one of the bioactive components isolated from *Phellinus linteus*, has been reported to possess anti-oxidant, anti-inflammatory and anti-proliferative properties, and exert protective effects against acute liver damage [12–14]. In addition, Hispolon has demonstrated antitumor effects in various cancer cells, including melanoma, leukemia, hepatocarcinoma, bladder cancer, and gastric cancer cells [15–19]. Collectively, these observations raise interest in

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investigating the potential of Hispolon to modulate ERα signaling in human breast cancer cells. In this study, we found that Hispolon significantly decreased expression of ERα and inhibited the growth of ER-positive human breast cancer cells. We suggest that Hispolon is a good candidate for use as an anticancer drug in chemoprevention and treatment of hormone-responsive breast cancer cells.

## 2. Materials and methods

### 2.1. Materials and reagents

Hispolon was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). MCF-7 and T47D cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/stereptomycin antibiotics. Antibodies for ERα, β-actin, pS2, PARP, Bcl-2 were purchased from Santa Cruz Biotechnology. The dual-luciferase reporter assay kit was from Promega (Madison, WI, USA).

### 2.2. Immunoblotting

MCF-7 and T47D cells were treated with Hispolon (0, 25, 50, μM) for 24 h, and treated with 100 μM Hispolon for various incubation time (0, 6, 12, 24 h). After lysing cells with RIPA buffer, ptoteins were resolved by SDS-PAGE and immunoblotted using primary antibodies such as anti-ERα and anti-β-actin antibody. After treatment with proper secondary antibodies, the immunoreactive bands were visualized by standard ECL method.

### 2.3. Immunofluorescence staining

MCF-7 and T47D cells were grown in 4-chamber slides in serum free media, and were treated with or without Hispolon (100 μM). After 24 h incubation, cells were fixed with 4% paraformaldehyde at 4 °C. Cells were washed with PBS containing 0.1% BSA and incubated with anti-ERα antibody for 1 h followed by 1 h incubation with fluorescence-tagged secondary antibody, then counterstained with DAPI for 5 min. Cell images were captured at 400× magnification on a Leica fluorescence microscope.

### 2.4. RT-PCR and quantitative real-time PCR

Total RNA isolation was performed with the use of the Trizol according to the manufacturer's protocol. The cDNA was synthesized with the use of 2 mg of total RNA through SuperScript reverse transcriptase (Bioneer, Daejon, South Korea) with oligo dT primers. PCR was done with a specific primer (ERa sense 5′-CGACGCCAGGGTGGCAGAGAAAGATT-3′; antisense 5′-GGCCAAAGGTTGGCAGCTCTC-ATGTC-3′; pS2 sense 5—TGCTGTTTCGACGACACCGTT-3′; antisense 5′-AGG-CAGATCCCTGCAGAAGT-3′). GAPDH, a non-regulated housekeeping gene was used as an internal control to normalize input cDNA. RT-PCR was performed using the Light-Cycler 480 using SYBR green master mix (Roche).

### 2.5. Luciferase reporter assay

MCF-7 and T47D cells were transiently co-transfected with 0.5 μg ERE or pS2 luciferase plasmid and 0.5 μg pSV-β-galactosidase

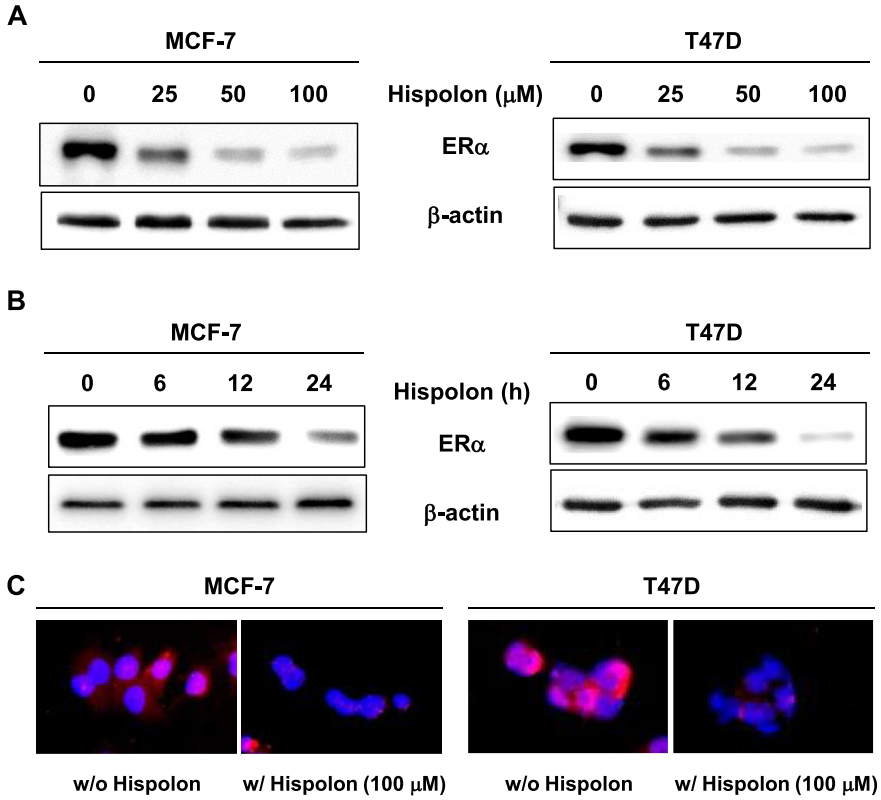


**Fig. 1.** Hispolon decreases ERα protein levels in MCF-7 and T47D cells. A and B, MCF-7 and T47D cells were treated with different concentrations of Hispolon for 24 h (A) or with 100 μM Hispolon for the indicated times (B). Cells were lysed, and proteins in lysates were resolved by SDS-PAGE and immunoblotted using anti-ERα and anti-β-actin antibodies. C, MCF-7 and T47D cells were grown in four-chambered slides and treated with 100 mM Hispolon for 24 h. Cells were fixed with paraformaldehyde, incubated with a polyclonal anti-ERα antibody for 1 h, and incubated with RITC-conjugated anti-mouse antibody for 1 h. The preparations were washed and counterstained with DAPI. Cell images were obtained using a Leica fluorescence microscope.

reporter vector using Lipofectamine 2000 (Invitrogen) transfection reagent. After transfection for 24 h, cells were treated with Hispolon (0, 25, 50, 100 μM). Cells extracts were prepared for the luciferase assays. The luciferase activity was normalized by β-galactosidase activity.

### 2.6. Cell proliferation and viability assays

All proliferation assays were based on the 3-[4,5-dimethythiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) method. Cells were seeded in a 96-well plate, $1 \times 10^4$ cells per well. After overnight culture, Hispolon was added to the cells and further cultured for 24 h. The media was removed and DMSO was added at MTT solubilization solution. Absorbance was measured at 550 nm.

### 2.7. Colony forming assay

Single-cell suspensions of $5 \times 10^3$ cells were seeded into 6-well plate and allowed to attach for 24 h at 37 °C in culture medium. Cells were then treated with 10 μM or 100 μM Hisplon. After 10 days, colonies were fixed with 100% methanol for 10 min at room temperature and stained with 0.1% crystal violet. Plates were washed with PBS and were photographed.

### 2.8. Statistical analysis

The results are presented as mean ± SE, and statistical comparisons between groups were carried out using one-way ANOVA followed by the Student's t-test. P-values of less than 0.05 were considered statistically significant.

## 3. Results

### 3.1. Hispolon downregulates ERα levels in breast cancer cells

To investigate the effect of Hispolon on ERα expression, we treated the ERα-positive human breast cancer cells, MCF7 and T47D, with different concentrations of Hispolon. Western blot analyses showed that Hispolon downregulated ERα protein levels in a concentration-dependent manner in both MCF-7 and T47D cells (Fig. 1A). An examination of MCF-7 and T47D cells treated with Hispolon at 100 μM for various durations further showed that Hispolon effects on ERα were time dependent, increasing as culture time increased (Fig. 1B). We also examined ERα expression in breast cancer cells using fluorescence imaging. Consistent with Western blotting results, ERα was hardly detectable after Hispolon treatment (Fig. 1C). Collectively, Western blotting and fluorescence imaging results suggest that Hispolon downregulates ERα protein expression in breast cancer cells. Given the observed decrease in

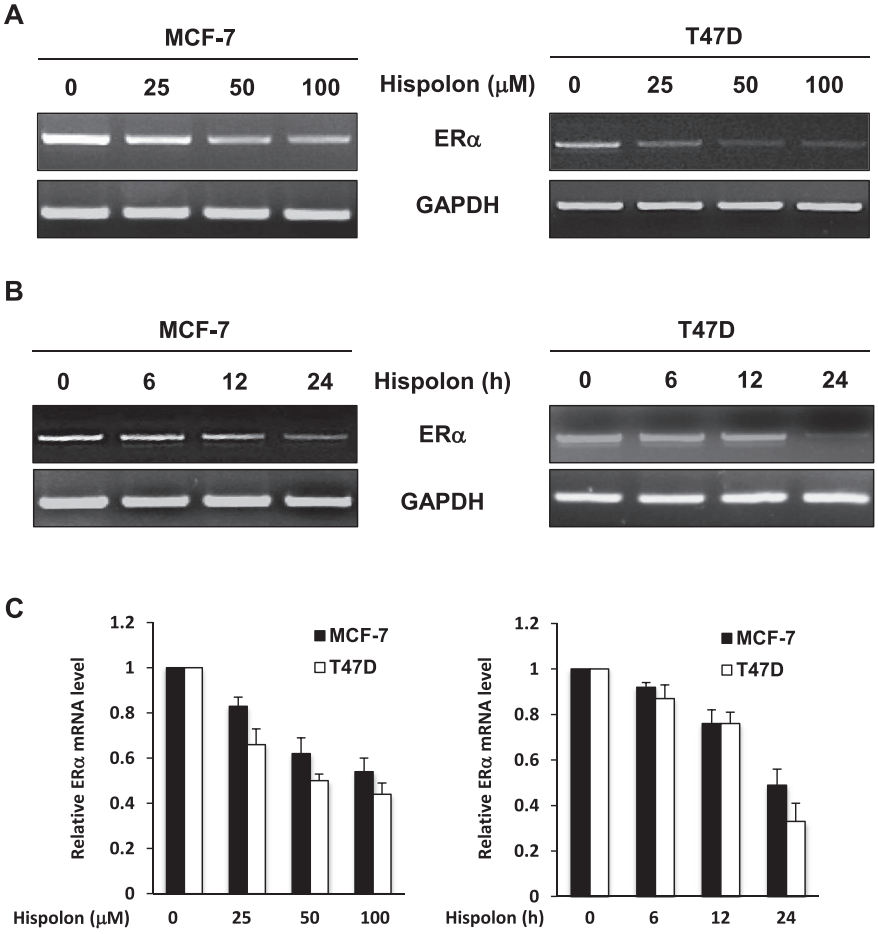


**Fig. 2.** Hispolon suppresses ERα mRNA expression in MCF-7 and T47D cells. A and B, MCF-7 and T47D cells were treated with different concentrations of Hispolon for 24 h (A) or with 100 μM Hispolon for the indicated times (B). Total RNA was extracted from cells, and RT-PCR analyses were performed using primers specific for ERα. The PCR products were separated on a 1.5% agarose gel; GAPDH was used as an internal control. C, Quantitative analysis of concentration- and time-dependent changes in ERα mRNA levels in Hispolon-treated MCF-7 and T47D cells. Results are expressed as means ± SD of triplicate measurements from a single experiment, representative of three separate experiments.

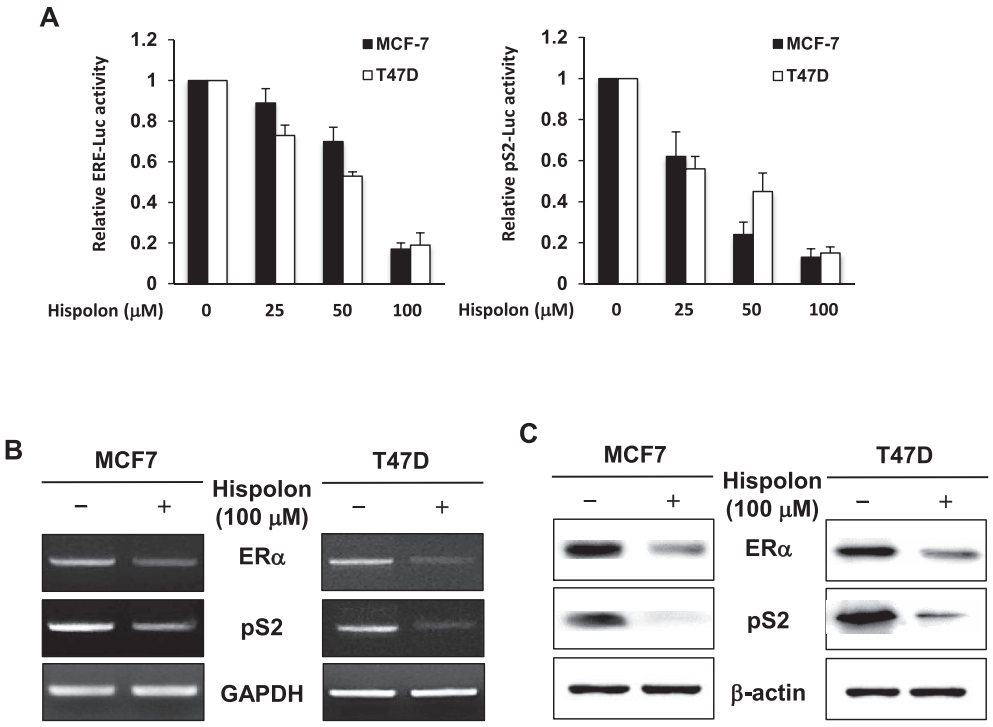


**Fig. 3.** Hispolon decreases the transcriptional activity of ERα. A, MCF-7 and T47D cells were transfected with an ERE- or pS2-luciferase reporter gene plasmid. After 24 h, cells were treated with the indicated concentration of Hispolon for 24 h and harvested for luciferase assays. Luciferase activity was normalized to β-galactosidase activity. Data are presented as relative luciferase activity, expressed as means ± SD of triplicate determinations. B and C, Hispolon suppresses mRNA and protein expression of the ER-responsive gene pS2. RT-PCR and immunoblot analyses were performed as described above.

ERα protein expression, we next examined whether Hispolon affected ERα expression at the mRNA level using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Hispolon decreased ERα mRNA levels in both a concentration- and time-dependent manner in both cell lines (Fig. 2). These results indicate that Hispolon decreases the expression of ERα at the transcriptional level.

### 3.2. Hispolon decreased the transcriptional activity of ER

ERα is an important transcription factor that regulates the expression of a variety target genes harboring the estrogen-response element (ERE) in their promoter [20,21]. Therefore, we examined the effect of Hispolon on the transcriptional activity of ERα̃ ER transactivation was determined by measuring luciferase activity in MCF7 and T47D cells transfected with an ERE-containing promoter-luciferase reporter gene. Hispolon decreased luciferase activity in a concentration-dependent manner; at 25 and 100 μM Hispolon, luciferase activity was 0.87- and 0.18-fold, respectively, of that in controls (Fig. 3A). To further investigate the Hispolon-induced decrease in ERα transcriptional activity, we examined expression of the ERα target gene pS2. MCF7 and T47D cells were transfected with a reporter construct driven by the pS2 promoter, and pS2 activation was determined by measuring luciferase activity. As shown in Fig. 3A, Hispolon decreased pS2-dependent luciferase activity in a concentration-dependent manner, effects similar to those observed with ERE-containing reporter constructs. We then examined Hispolon effects on the expression of pS2 mRNA and protein. In both MCF7 and T47D cells, Hispolon induced a decrease in pS2 at both mRNA and protein levels (Fig. 3B and C). These results indicate that Hispolon decreases the transcriptional activity of ERα, leading to suppression of the expression of the representative target protein pS2.

### 3.3. Hispolon inhibits the growth of human breast cancer cells

ERα is an important regulator of the growth of estrogen-dependent breast cancer cells. As shown above, Hispolon suppressed ERα expression at the transcriptional level and subsequently inhibited the transcriptional activity of ERα, resulting in inhibition of cell-proliferation regulators in the ER-positive breast cancer cells, MCF7 and T47D. To examine the effect of Hispolon on proliferation of both cell types, we first measured the levels of proteins involved in apoptosis after incubating with or without 100 μM Hispolon for 24 h. Hispolon increased levels of cleaved poly ADP-ribose polymerase (PARP), an apoptotic marker, while decreasing expression of the anti-apoptotic molecule Bcl-2 (Fig. 4A). To determine the drug concentration that causes 50% growth inhibition ($IC_{50}$), we treated cells with different concentration of Hispolon for 24 h, and measured cell viability by MTT (3-{4,5-dimethythiazol-2-yl}-2,5-diphenyl tetrazolium bromide) assay. As shown in Fig. 4B, $IC_{50}$ values for both cell types were very similar (~70 μM). The long-term effects of Hispolon were determined by culturing MCF-7 and T47D cells with or without Hispolon for 10–15 d and then performing colony-forming assays. At a concentration of 10 μM, Hispolon showed a slight inhibitory effect, whereas 100 μM Hispolon almost completely inhibited colony formation (Fig. 4C).

## 4. Discussion

Breast cancer is one of the most common cancers and the leading cause of cancer-related death in women [22]. Approximately 70% of breast cancer cells overexpress ERs, referred to as ER-positive cells [23]. In addition, ERα influences cell division, cell proliferation and DNA replication, and thus stimulates the rapid growth of cancer [24]. Therefore, control of ERα levels and the

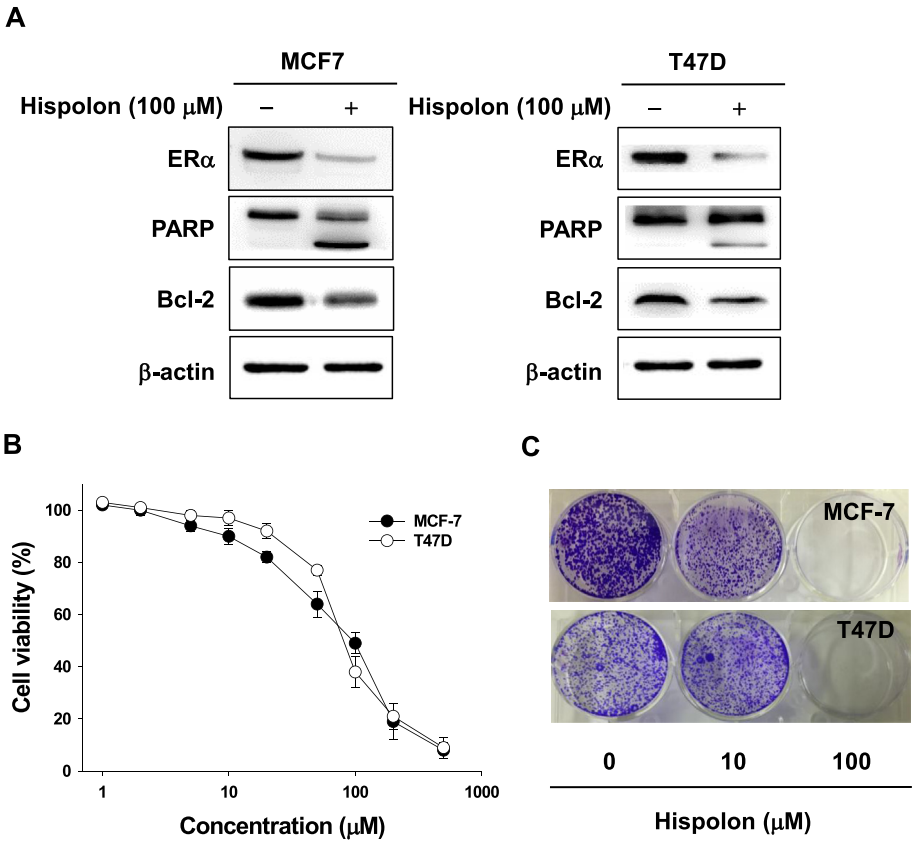


**Fig. 4.** Hispolon inhibits the growth of MCF-7 and T47D cells. A, Immunoblot analysis of PARP (pro-apoptotic factor) and Bcl-2 (anti-apoptotic factor) in MCF-7 and T47D cells after treating with 100 μM Hispolon for 24 h. B, MCF-7 and T47D cells were seeded in a 96-well plate and treated with different concentrations of Hispolon for 24 h. Cell proliferation was determined by MTT assay. Data are presented as the percentage of proliferation relative to that in medium-treated controls. All measurements were performed in triplicate. C, MCF-7 and T47D cells were seeded in a 6-well plate and treated with 0, 100, or 500 mM Hispolon for 15 d. Colonies were fixed with 100% methanol for 10 min at room temperature and stained with 0.1% crystal violet. Representative photographs demonstrating colony formation are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

transcriptional activity of ERα have been highlighted in breast cancer development. Currently, ER-targeting drugs that act through various mechanisms, such as blocking the activity of ERs, inhibiting the production of endogenous estrogen and reducing the stability of the ER protein, are widely used in breast cancer treatment [21].

Hispolon, derived from *Phellinus linteus*, is known for its anticancer properties, but the mechanism underlying this activity is not understood. Here, we examined the effects of Hispolon on the expression of ERα and growth of the ER-positive human breast cancer cell lines, MCF-7 and T47D. Consistent with Hispolon inhibition of ERE-dependent transcriptional activity in ER-positive human breast cancer cells, Hispolon showed anticancer activity in these cells. We further found that Hispolon reduced the expression of ERα at mRNA as well as protein levels. Hispolon-induced decreases in ERα protein levels led to repression of ERα transcriptional activity, which resulted in a significant reduction in the expression of the ER-responsive gene pS2—the main gene presenting in ER-expressing breast cancer tissues and an important prognostic indicator [25]. We also found that Hispolon increased PARP cleavage, indicating enhanced apoptosis, and reduced expression of Bcl-2, a typical survival factor. Although further *in vivo* studies are needed to establish the potential of Hispolon as an anticancer drug for treatment of breast cancer, our results taken together suggest that Hispolon is an effective inhibitor of breast cancer development in ER-positive human breast cancer cells.

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## Transparency document

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