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4-(E)-{(p-tolylimino)-methylbenzene-1,2-diol}, 1 a novel resveratrol analog, differentially regulates estrogen receptors α and β in breast cancer cells

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Abstract

Breast cancer is a public health concern worldwide. Prolonged exposure to estrogens has been implicated in the development of breast neoplasms. Epidemiologic and experimental evidence suggest a chemopreventive role of phytoestrogens in breast cancers. Resveratrol, a naturally occurring phytoestrogen, has been shown to have potent anti-cancer properties. However, poor efficacy and bioavailability have prevented the use of resveratrol in clinics. In order to address these problems, we have synthesized a combinatorial library of azaresveratrol analogs and tested them for their ability to inhibit the proliferation of breast cancer cells. We have recently shown that 4-(E)-{(p-tolylimino)-methylbenzene-1,2-diol} (TIMBD), has better anti-cancer properties than resveratrol and any other resveratrol analog we have synthesized so far. The objective of this study was to investigate the regulation of estrogen receptors (ERs) α and β by TIMBD in breast cancer cell lines. We demonstrate that TIMBD significantly induces the mRNA and protein expression levels of ER β and inhibits that of ER α . TIMBD inhibits mRNA and protein expression levels of oncogene c-Myc, and cell cycle protein cyclin D1, which are important regulators of cellular proliferation. TIMBD significantly induces protein expression levels of tumor suppressor genes p53 and p21 in MCF-7 cells. TIMBD inhibits c-Myc in an ER β -dependent fashion in MCF-10A and ER β 1-transfected MDA-MB-231 cells, suggesting regulation of ERs as an important upstream mechanism of this analog. ER β plays a partial role in inhibition of proliferation by TIMBD while ER α overexpression does not significantly affect TIMBD's inhibition.

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Keywords

Estrogen receptors; Resveratrol analogs; TIMBD; Breast cancer inhibition

Introduction

Epidemiological evidence suggests that diet and nutrition may play an important role in breast cancer development [1–3]. Studies have shown that Asian women have three times lower risk for breast cancer than women living in the United States [4]. This lower risk has been partially associated with Asian diets that consist of considerably higher amounts of phytoestrogens [1, 5, 6]. Phytoestrogens are biologically active metabolites of plant origin that structurally mimic mammalian estrogens and are mainly found in fruits, vegetables and herbs [1–3].

Resveratrol (Res), a naturally occurring phytoalexin obtained from plants, has been shown to have both chemo-preventive and anti-cancer activities [7, 8]. Resveratrol is considered a phytoestrogen since it can bind to and activate estrogen receptors (ERs) α and β [9]. The significance of ERs in breast cancer is underscored by the fact that 75% of breast cancer cases are ER-positive and depend on endogenous estrogens for tumor progression [10, 11, 12]. Since Res structurally mimics estrogens, it acts as partial agonist at both the ERs [9, 13]. However, in presence of estrogens, which are stronger agonists at ERs, Res acts as an antagonist to ER α , although no antagonist activity for ER β has been reported [9, 13]. The partial agonist/antagonist activity of Res depends on many factors including concentration, relative ER expressions, cofactors and cell-type [9]. Resveratrol has shown a substantially higher binding affinity and transcriptional activation for ER β compared to ER α suggesting it may be able to activate ER β at concentrations lower than that required to activate ER α [9, 14]. Studies have reported that regulation of estrogen signaling by Res can affect cellular proliferation in breast epithelial and breast cancer cells [13, 15]. It has been postulated that the growth inhibitory effects of Res could be attributed to its higher transcriptional activity at ER β , given the inhibitory role of ER β on estrogen-mediated cellular proliferation [16, 17]. Phytoestrogens are known to show a concentration dependent biphasic effect on cellular proliferation with growth promoting effects seen at lower concentrations and inhibitory effects seen at higher concentrations [9, 13, 18]. Resveratrol has been shown to stimulate growth of ER-positive MCF-7 breast cancer cells at low concentration of 4 μ M and inhibit growth at higher concentration of 44 μ M [19, 20]. The growth inhibitory effects of Res have also been attributed to other mechanisms including regulation of cell cycle and induction of tumor suppressor proteins, which may be independent of interactions with ERs [21].

Several *in vivo* studies have shown the chemo-preventive and anti-cancer effects of Res [15]. Resveratrol has been shown to inhibit estrogen-induced carcinogenesis in rats [22]. Resveratrol has also shown inhibition of 7,12-dimethylbenz(a)anthracene (DMBA) and N-methyl-N-nitrosourea (MNU)-induced mammary carcinomas in rats [23, 24]. Resveratrol induced apoptosis and inhibited angiogenesis in *in vivo* xenograft studies with MDA-MB-231 cell line [25]. However, in 4T1 breast cancer cells, the inhibitory effect of Res seen *in vitro* studies was lost when the experiment was conducted *in vivo* in mice [26]. Despite

the ample data available on Res's anticancer effects *in vitro* and in animals, clinical support in human studies has been lacking [15]. The primary reason for this is the poor bioavailability of Res in humans [15]. Pharmacokinetic studies in healthy human subjects have revealed that Res is extensively metabolized in the liver with an oral bioavailability of less than 1% [27, 28].

As reported recently, we have synthesized a library of azaresveratrol analogs that possess the basic structure of Res with additional pharmacophoric groups in an attempt to increase the anti-cancer efficacy of Res [29]. Of the six analogs tested, two analogs referred to as 3e and 3b; now termed as HPIMBD and TIMBD, respectively, showed better cancer inhibitory activities than Res [29]. 4-(E)-{(p-tolylimino)-methylbenzene-1,2-diol} (TIMBD) showed better potency than any of the other analogs in inhibiting the proliferation of breast cancer cell lines [29]. We have recently shown the effect of 4-(E)-{(4-hydroxyphenylimino)-methylbenzene, 1,2-diol} (HPIMBD) in regulating ERs in breast cancer cells [30]. In the present study, we investigated the effect of TIMBD on the regulation of ER α and β . We present evidence that TIMBD significantly induces the mRNA and protein expression levels of ER β and inhibits that of ER α . We further demonstrate that TIMBD significantly inhibits mRNA and protein expression levels of oncogene c-Myc and cell cycle protein cyclin D1. Further, TIMBD induces protein expression levels of tumor suppressor genes p53 and p21 in MCF-7 breast cancer cells. TIMBD regulates the expression of c-Myc in an ER β -dependent fashion in MCF-10A and ER β 1-transfected MDA-MB-231 cells. However, silencing ER β in MDA-MB-231-ER β transfected cells only partially rescues cells from inhibition by TIMBD. Overexpression of ER α in ER α -negative MDA-MB-231 cell line does not significantly affect the inhibitory potential of TIMBD. Taken together, our studies suggest that TIMBD, a novel analog of Res, differentially regulates the expressions of ERs and inhibits proliferation of breast cancer cells via targeting multiple molecular mechanisms.

Materials and Methods

Chemicals

Resveratrol was purchased from Sigma-Aldrich (St. Louis, MO). Resveratrol analog TIMBD was synthesized and purified by our group as reported previously [29]. Doxycycline was purchased from Clontech (Mountain View, CA). Resveratrol and TIMBD were dissolved in dimethyl sulfoxide (DMSO) prior to treatments. Doxycycline was dissolved in sterile purified water. The concentration of DMSO in control experiments was always 1/1000th (vol/vol) of the final medium volume. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO). A stock solution of MTT reagent was prepared by dissolving MTT in sterilized PBS to a final concentration of 1 mg/ml.

Cell Culture

Non-neoplastic breast epithelial cell line MCF-10A and breast cancer cell lines MCF-7, T47D and MDA-MB-231 were purchased from ATCC (Manassas, VA). Estrogen receptor β 1-transfected MDA-MB-231 and empty vector-transfected MDA-MB-231 were a gift from Dr. Leigh C. Murphy (University of Manitoba, Canada). All breast epithelial and breast

cancer cell lines were cultured as reported recently [30]. Cells were treated with 50 μ M doses of Res or TIMBD for up to 72 hours. Cells from ER β 1-transfected and empty vector-transfected MDA-MB-231 were treated with doxycycline (final concentration 1 μ g/ml) for 24 hours prior to treatment with Res or TIMBD, to induce the expression of ER β from the Tet-On inducible expression vector. Following treatments, cells were lysed to harvest either RNA or proteins.

MTT cell viability assay

Cells were seeded in 96-well plates for MTT assays and treated with 50 μ M Res or TIMBD for 72 hours [30]. After treatments, cells were incubated with MTT reagent at 37°C. Formazan crystals were dissolved in DMSO and plates were read in Benchmark Plus microplate spectrophotometer (Biorad, Hercules, CA). Percentage of viable cells were calculated as described recently [30].

Lactate Dehydrogenase release cytotoxicity assay

This assay was performed using a cytotoxicity LDH assay kit from Pierce (Thermo Fisher Scientific, Rockford, IL). Cells were plated in 96-well plates and treated with different doses of Res or TIMBD as described recently [30]. Post-treatments, medium was collected and absorbance was measured using the Benchmark Plus microplate spectrophotometer (Biorad, Hercules, CA). Mean % released LDH compared to total intracellular LDH was calculated as described recently [30].

BrdU Incorporation Assay

The Bromodeoxyuridine (BrdU) incorporation assay was carried out using the FITC BrdU Flow Kit (BD Biosciences, San Jose, CA). Cells were plated in 6-well plates and treated with 50 μ M Res or TIMBD for 48 hours [30]. BrdU incorporating cells were analyzed following manufacturer's instructions and as reported recently [30]. The results are expressed as the % of cells incorporating BrdU and calculated as mean $\pm$ SEM from three different experiments.

Reverse transcription and Real-time PCR

Following treatments with Res or TIMBD, total RNA was isolated using TRI reagent and reverse transcribed using superscript II reverse transcription system as described recently [30]. Real-time PCR was performed using human specific ER α (QT00044492) and ER β (QT00060641) primers (Qiagen, Valencia, CA). The expression levels of ER α or β relative to cyclophilin were determined as reported recently [30]. A standard curve was run on each plate to allow for accurate quantification of cDNA, as reported previously [31–37].

Western Blot analysis

Western blot analyses were performed as described previously [31–37]. Affinity purified rabbit polyclonal antibodies against ER α (Santa Cruz, sc-543), ER β (Santa Cruz, sc-8974), c-Myc (Santa Cruz, sc-788), cyclin D1 (Santa Cruz, sc-753), p53 (Santa Cruz, sc-55476) and p21 (Santa Cruz, sc-6264) were diluted in PBS/0.05% Tween-20 and used for immune-detection as reported recently [30]. Following incubation with primary antibodies,

membranes were incubated with the respective secondary antibodies for each protein as described recently [30]. Chemiluminescent detection was performed using the BM Chemiluminescence Detection kit (Roche, Indianapolis, IN) and the FluorChem HD2 Imaging system (Alpha Innotech Corporation, San Leandro, CA), with AlphaEaseFC Image Analysis software (Alpha Innotech Corporation, San Leandro, CA). Membranes were then stripped with Restore Western blot stripping buffer (Thermo Scientific, Rockford, IL) and re-incubated with α -tubulin mouse monoclonal antibody (Santa Cruz, sc-53030) as the loading control.

RNA interference

Small interfering RNAs (siRNAs) for ER β (sc-35325) and control siRNA (sc-37007) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). MCF-10A, ER β 1-transfected MDA-MB-231, MCF-7 and T47D cells were transfected with 1 nmol/l siER β using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) as described previously [31–34]. Cells transfected with siER β were treated with 50 μ M TIMBD for 12 hours (ER β 1-transfected MDA-MB-231), 24 hours (MCF-10A) and 48 hours (MCF-7 and T47D) and used for western blot analyses.

Transient transfection

Estrogen receptor α and empty vector plasmids (V864-20) were obtained from Invitrogen (Carlsbad, CA). MDA-MB-231 cells were transiently transfected with ER α and empty-vector plasmids using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) for 48 hours. Media was changed and cells were allowed to grow for another 24 hours. Cells were then harvested, counted and plated for further assays. Western blot analyses were performed to confirm ER α expression.

Clonogenic cell survival assay

500 viable cells were seeded in 6-well plates and allowed to grow for 24 hours in phenol red-free complete media. The cells were then treated with either vehicle (DMSO) or TIMBD for 72 hours after which they were washed in PBS and incubated for an additional 8 days in complete medium. The colonies obtained were fixed in 10% formalin for 15 min followed by staining with 0.1% crystal violet solution [22, 31, 33]. The colonies were counted, photographed and compared with respective untreated cells. Each treatment was done in triplicate.

Mammosphere assay

Mammosphere formation assays were performed using ultra-low attachment plates (Corning, Lowell, MA). 5000 viable cancer cells transiently transfected with siER β , control siRNA or un-transfected cells were seeded into 24-well plates. Cells were grown in serum-free DMEM/F-12 (50:50) medium supplemented with 1 $\times$ B27 (Invitrogen), 20 ng/ml epidermal growth factor (Invitrogen), 20 ng/ml basic fibroblast growth factor (Invitrogen), 1 μ g/ml hydrocortisone (BD Biosciences, Bedford, MA), 5 μ g/ml insulin (Invitrogen), 0.1% penicillin/streptomycin (Lonza, Walkersville, MD) and 4 μ g/ml heparin calcium salt (Thermo Scientific). Cells were treated with vehicle or TIMBD. After 3–5 days of

incubation, mammospheres were viewed under the microscope and photographed [22, 31, 33]. Three replicate wells from a 24-well plate were used for each experimental condition.

Statistical analysis

Statistical analyses were performed using Sigma Plot 11.0 software (Systat Software, San Jose, CA) and IBM SPSS Statistics 19 software (IBM, Armonk, NY). Student's t-test and one-way analysis of variance (ANOVA) with a least significant difference (LSD) post-hoc test were used to compare ER α , ER β , c-Myc and cyclin D1 mRNA expression changes and ER α , ER β , c-Myc, cyclin D1, p53 and p21 protein expression changes in treated cells as described recently [30]. These tests were also used to compare the changes in percent proliferation of cells, percent release of LDH, percent BrdU incorporation and number of colonies formed in experiments. A p-value < 0.05 was considered significant. All experiments were performed in triplicates or quadruplicates.

Results

TIMBD significantly inhibits proliferation of breast cancer cell lines

We have previously reported the effect of Res, TIMBD and other Res analogs on cellular proliferation of non-neoplastic breast epithelial cell line MCF-10A and breast cancer cell lines MDA-MB-231 and T47D [29]. TIMBD exhibited most potent inhibitory activity against MDA-MB-231 cells (with ~75% inhibition of cellular proliferation) and T47D cells (with ~60% inhibition) compared to Res which induced only 40% inhibition in both cell lines [29]. Also, TIMBD did not affect the growth of MCF-10A cells up to 50 μ M concentration suggesting it is not toxic to normal breast cells and selective towards cancer cells [29]. We further evaluated the growth inhibitory effect of TIMBD on three other breast cancer cell lines, namely, MCF-7, vector-transfected and ER β 1-transfected MDA-MB-231 cell lines. We have previously reported that Res treatment resulted in about 40 – 50% inhibition of cellular proliferation in the above mentioned cell lines [29, 30]. In the current study, we demonstrate that TIMBD treatment results in about ~70% inhibition of MCF-7 cells and >70% inhibition of the vector-transfected and ER β 1-transfected MDA-MB-231 cells (Fig. 1a). TIMBD was better than HPIMBD in inhibiting cellular proliferation in these breast cancer cells, results of HPIMBD have been reported previously [30].

Dose and time-dependent cytotoxicity induced by Res analog TIMBD in breast cancer cell lines

Breast cancer cell lines MCF-7, T47D, MDA-MB-231, vector-transfected and ER β 1-transfected MDA-MB-231 were treated with different doses of TIMBD (0, 6.25, 12.5, 25 and 50 μ M) for up to 72 hours. The extent of cell death was measured after 24, 48 and 72 hours. TIMBD (50 μ M dose) significantly resulted in ~30% increase in LDH release in MCF-7 and T47D cells and ~40% increase in the triple-negative MDA-MB-231, vector-transfected and ER β 1-transfected MDA-MB-231 cell lines after 72 hours treatment (Fig. 1b). Treatment of MCF-10A cells with different doses of TIMBD (0, 6.25, 12.5, 25 and 50 μ M) did not have any cytotoxic effect up to a dose of 50 μ M (Fig. 1c). A time-dependent study showed that TIMBD significantly increased cytotoxicity at 48 hours which further increased after 72 hours in all the tested breast cancer cell lines (Fig. 1d–h). A dose-

dependent study suggested that TIMBD caused significant cytotoxicity at 25 and 50 μM doses (Fig. 1d–h). The cytotoxic effects induced by TIMBD were comparable to that of HPIMBD reported previously [30].

TIMBD significantly decreases BrdU incorporation in breast cancer cell lines

To assess the effect of TIMBD on cellular proliferation, non-neoplastic breast epithelial and breast cancer cell lines were treated with 50 μM TIMBD for 48 hours and BrdU incorporation was assessed. TIMBD did not affect the cellular proliferation of MCF-10A cells at 50 μM dose (Fig. 1i). However, TIMBD caused a significant decrease in the percentage of breast cancer cell lines incorporating BrdU (Fig. 1i). As reported previously [30], the vehicle treated control cells of all the cell lines tested showed about 20–30% of BrdU incorporation. TIMBD treatment resulted in about 2–5% of cancer cells incorporating BrdU, which amounts to 80–90% decrease in BrdU incorporation compared to respective controls (Fig. 1i). BrdU incorporation studies confirmed that TIMBD is better than HPIMBD, which showed 75–85% inhibition in cellular proliferation of breast cancer cells [30].

TIMBD significantly induces mRNA and protein expression levels of ER β in breast cancer cells

Breast epithelial and breast cancer cell lines mentioned above were treated with 50 μM Res or TIMBD for up to 72 hours and PCR analyses were performed. Please note that the data on the induction of ER β by Res have been reported previously [30]. In this study, we demonstrate that TIMBD significantly induced ER β mRNA levels in all cell lines tested compared to Res or control treated cells (Fig. 2a). TIMBD treatment resulted in 2–3-fold induction of ER β mRNA in MCF-7, T47D and MDA-MB-231 cell lines (Fig. 2a). TIMBD significantly induced protein expression levels of ER β , ranging from 2 to 3-fold, in all the breast cancer cell lines tested and modestly increased ER β protein expression in MCF-10A cells up to 1.2-fold (Fig. 2b). The ER β 1-transfected MDA-MB-231 cell line also showed induction of ER β 1 upon TIMBD treatment (Fig. 2b). The ER β 1 transgene in this cell line is under the rTA-regulated promoter and not its natural promoter [30]. A time-course study with TIMBD showed a maximal induction of ER β after 48 hours in both MCF-7 and T47D cell lines; and after 12 hours in MDA-MB-231 cell line (Fig. 2c). A dose-response study showed a maximal induction of ER β protein at a dose of 50 μM (Fig. 2d). With respect to ER β induction, effect of TIMBD was comparable to that of HPIMBD, reported previously [30].

TIMBD significantly inhibits mRNA and protein expression levels of ER α in breast cancer cells

MCF-7 and T47D cells which express ER α [38] were treated with 50 μM Res or TIMBD for up to 72 hours. TIMBD significantly inhibited mRNA and protein expression of ER α in MCF-7 and T47D cell lines compared to respective controls (Fig. 3a and b). The protein inhibition was 80–90% in T47D and 50% in MCF-7 cell lines (Fig. 3b). A time-course study showed maximal inhibition of ER α protein expression levels at 48 hours post-TIMBD treatment (Fig. 3c). A dose-response study showed a dose-dependent inhibition of ER α expression levels with maximal inhibition of 90% at 100 μM dose in T47D cells (Fig. 3d).

With regards to ER α inhibition, effect of TIMBD treatments was comparable to HPIMBD [30]. The ratio of ER β :ER α was determined as a ratio of fold protein expressions in MCF-7 and T47D cells after 48 hours of treatment with 50 μ M TIMBD. TIMBD showed a significant increase in ER β :ER α ratio up to 13-fold (Table 1) as compared to a 10-fold increase by HPIMBD in T47D cell line, as reported previously [30].

TIMBD inhibits mRNA and protein expression levels of c-Myc and cyclin D1 in breast cancer cells

Breast epithelial and breast cancer cell lines were treated with 50 μ M TIMBD for up to 72 hours to assess expression levels of oncogene c-Myc and cell cycle regulating protein cyclin D1 [39]. TIMBD significantly inhibited c-Myc and cyclin D1 mRNA levels in MCF-7, T47D and MDA-MB-231 cell lines up to 50% compared to control cells (Fig. 4a and c). TIMBD significantly inhibited protein expression levels of c-Myc in all the tested breast cancer cell lines, ranging from 50–70% and up to 30% in MCF-10A cells (Fig. 4b). TIMBD significantly inhibited protein expression levels of cyclin D1 in all the tested breast cancer cell lines, ranging from 50–70% and up to 30% in MCF-10A cells (Fig. 4d). The effect of TIMBD on expression of c-Myc and cyclin D1 was comparable to that of HPIMBD [30].

TIMBD induces protein expression levels of tumor suppressor genes, p53 and p21, in MCF-7 breast cancer cells

In order to assess the effect of Res analog TIMBD on p53 and p21, MCF-7 cells, which express wild-type p53 [40], were treated with 50 μ M TIMBD. TIMBD significantly induced protein expression levels of both p53 and p21 in MCF-7 breast cancer cells (Fig. 5a). TIMBD showed a significant induction of both p53 and p21 protein expression levels up to 5-fold (Fig. 5a). A time-course study showed significant induction in protein expression levels of p53 and p21 in MCF-7 cells up to 24 hours peaking at 6 hours (Fig. 5b). Compared to HPIMBD, which showed 2-fold induction in p53 and p21 [30], TIMBD showed a substantial increase up to 5-fold, supporting its better inhibitory potential.

TIMBD inhibits protein expression of oncogene c-Myc in an ER-dependent fashion

On lines similar to HPIMBD treatment reported previously [30], we investigated whether TIMBD inhibits c-Myc in an ER β -dependent fashion. MCF-10A, ER β 1-transfected MDA-MB-231, MCF-7 and T47D cell lines were transfected with siER β for 48 hours, followed by treatment with TIMBD for 12 hours (ER β 1-transfected MDA-MB-231), 24 hours (MCF-10A) or 48 hours (MCF-7 and T47D). The treatment time points with TIMBD are based on maximal induction time of ER β for the respective cell lines following TIMBD treatment. Treatment with TIMBD following siER β transfection failed to inhibit c-Myc protein expression in MCF-10A and ER β 1-transfected MDA-MB-231 cells, suggesting that inhibition of c-Myc occurs by an ER β -dependent mechanism in these cells (Fig. 6a). However, this observation did not hold true for MCF-7 and T47D cells, suggesting that TIMBD may inhibit c-Myc in these two cell lines in an ER β -independent fashion (Fig. 6a). This effect is most likely to happen via a direct inhibition of ER α as it has been reported that ER α regulates c-Myc expression in breast cells [41].

TIMBD binds ER β with high affinity

Docking studies were carried out with TIMBD using protocols reported previously [29, 30]. TIMBD was found to dock into the active site of ER β protein cavity with two hydrogen bonding interactions (Fig. 6b and Table 2). TIMBD exhibited a binding energy of -8.3 Kcal/mol in ER β protein cavity. The vicinal hydroxyl groups of TIMBD were found to interact with GLY472 (2.0 Å) and HIS475 (2.1 Å) via hydrogen bonding in the ER β cavity. TIMBD showed binding energy and hydrogen bonding interactions comparable to Res which has been reported previously (Table 2) [30].

TIMBD partially inhibits proliferation of MDA-MB-231-ER β transfected cell line in an ER β dependent fashion

Colony formation and mammosphere formation assays were carried out in MDA-MB-231-ER β cell line in order to study the role of ER β in inhibition of cellular proliferation by TIMBD. MDA-MB-231-ER β cells were transfected with siER β for 48 hours followed by treatment with TIMBD for 12 hours. Silencing ER β partially restored the colony and mammosphere forming ability of cells treated with TIMBD (Fig. 7a and b). This suggests that TIMBD may have multiple molecular targets such that even if ER β is silenced, other potential targets may compensate for the inhibitory effect.

TIMBD inhibits proliferation of both MDA-MB-231 and ER α -transfected MDA-MB-231 cells

We also assessed the effect of ER α expression in an ER α -negative cell line on TIMBD's inhibitory properties. MTT proliferation and colony formation assays were performed on MDA-MB-231 cells transiently transfected with either ER α or empty vector plasmids. Following transfection, cells were treated with 50 μ M TIMBD for an additional 48 hours. Expression of ER α in MDA-MB-231 cells increased proliferation by 31% compared to vector-only-transfected cells as determined by MTT assays (Fig. 8a). A similar increase of 34.6% in number of colonies was observed in ER α -transfected MDA-MB-231 cells compared to vector-transfected cells (Fig. 8b). TIMBD treatment resulted in 38.5% inhibition in vector-only-transfected and 37% inhibition in ER α -transfected MDA-MB-231 cells when compared to respective vehicle controls of the same cell type in MTT proliferation assays (Fig. 8a). Colony formation data showed a 55.4% and 48.3% inhibition in number of colonies of TIMBD treated ER α -transfected and vector-transfected MDA-MB-231 cells, respectively, compared to vehicle-treated controls of the same cell type (Fig. 8b).

Discussion

A logical approach to overcoming poor bioavailability and lack of specificity of Res is to modify the chemical structure of Res and use alternative pharmacophoric groups in accordance with structure activity relationship studies [29]. Various analogs with hydroxy-, acetate-, methyl, methoxy-, fluoro-, and methyl ether- substituents have been synthesized and tested for their effects in various cancer cell lines [42]. For example, Res analogs with 3,4-dihydroxy substituents on A-ring have shown the most potent apoptosis inducing properties [43,44]. Thus, the azaresveratrol analogs were synthesized in such a way that the

3,4-dihydroxy substitution was maintained and aza functionality was introduced to make the compound resistant to metabolism [29].

Among the six synthesized analogs, TIMBD demonstrated better potency than Res and any other analog, in selectively inhibiting the growth of breast cancer cells [29, 30]. We have previously shown that the other active analog, HPIMBD, showed differential regulation of ERs in breast cancer cells [30]. In this study, we have demonstrated that TIMBD also shows differential regulation of ERs. TIMBD induces mRNA and protein expression levels of ER β and inhibits that of ER α . TIMBD also inhibits mRNA and protein expression levels of oncogene c-Myc and cyclin D1, which regulates the cell cycle, suggesting that affecting cell cycle regulators may be one mechanism of inhibition of cellular proliferation by TIMBD (Fig. 4). TIMBD also induces the protein expression levels of the tumor suppressor proteins p53 and p21 (Fig. 5). P53 induction suggests that TIMBD treatment may lead to cell cycle arrest/apoptosis in cells that express wild-type p53, such as MCF-7 cells. However, all other cell lines, except MCF-7 and MCF-10A, express mutant forms of p53, suggesting that there are other potential targets that result in TIMBD's inhibition of cancer cells. Next, we wanted to dissect the mechanistic pathway linking changes in estrogen receptor expression to cellular proliferation. To determine if the effect on inhibition of c-Myc was a result of differential regulation of ERs, we silenced ER β in ER α -positive and negative cells followed by treatments with TIMBD (Fig. 6a). TIMBD was not able to inhibit the protein expression of c-Myc in the absence of ER β (Fig. 6a) suggesting that ER β may play an important role in inhibition of c-Myc and thus breast cancer cell proliferation by TIMBD. However, this effect of ER β was observed in MCF-10A and ER β 1-transfected MDA-MB-231 cell lines but not in ER α expressing MCF-7 and T47D cells, suggesting that the regulation of c-Myc in ER α -positive cells occurs in an ER β -independent fashion (Fig. 6a). To show that the effect on inhibition of c-Myc could translate to inhibition of cellular proliferation, colony formation and mammosphere assays were performed. However, unlike the significant results seen in terms of c-Myc expression, results from colony formation and mammosphere assays suggested a partial role for ER β in TIMBD's inhibition. Silencing ER β followed by TIMBD treatment in MDA-MB-231-ER β cells did not significantly affect the number of colonies but affected the size of colonies formed and thus, intensity of crystal violet staining (Fig. 7a). Mammosphere data showed that silencing ER β was only partially able to rescue cells from inhibition by TIMBD (Fig. 7b). This suggests that there may be other mechanisms through which TIMBD is able to inhibit proliferation in the absence of ER β , which is evident from our studies that have shown multiple molecular targets for TIMBD. Also, being an analog of Res, which itself has multiple molecular targets, TIMBD's multiple pathway regulation in cells was not unexpected. Next, we studied the role of ER α in TIMBD's inhibitory mechanism. Transient transfection of MDA-MB-231 cells with ER α increased the proliferation of MDA-MB-231 cells compared to vector-controls. Treatment of both vector and ER α -transfected cells with TIMBD resulted in proportionally equal inhibition of proliferation compared to vehicle-treated controls of the same cell type in MTT assays. A similar experiment done using colony formation assay resulted in 7% decrease in inhibition by TIMBD of ER α -transfected compared to vector-transfected MDA-MB-231 cells. This result suggests that overexpression of ER α did not significantly affect the extent of inhibition by TIMBD. Docking of TIMBD in the ER β protein cavity showed comparable

binding interactions as shown by the parent compound Res (Fig. 6b and Table 2). TIMBD (binding energy -8.3 Kcal/Mol) showed two hydrogen bonding interactions (GLY472 and HIS475) in ER β protein cavity when compared to Res (binding energy -8.2 Kcal/Mol) which showed three hydrogen bonding interactions in the cavity. Taken together, our studies indicate that, TIMBD, a novel analog of Res, differentially regulates expressions of ERs α and β and cellular proliferation markers in breast cancer cells. Regulation of ERs may be an important mechanism for inhibition of cancer cell growth. TIMBD may thus be potentially useful in the therapy and/or prevention of breast cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Resveratrol analog TIMBD inhibits growth of breast cancer cells
- TIMBD induces protein expression levels of ER β and inhibits that of ER α
- TIMBD inhibits c-Myc and cyclin D1, and induces p53 and p21
- TIMBD suppresses c-Myc in an ER-dependent fashion

Fig. 1a

Fig. 1b

Fig. 1c

Fig. 1d

Fig. 1e

Fig. 1f

Fig. 1g

Fig. 1h

Fig. 1i

Fig. 1. TIMBD significantly inhibits proliferation of breast cancer cell lines and shows a dose- and time-dependent cytotoxicity

a) Non-neoplastic breast epithelial cell line MCF-10A and breast cancer cell lines MCF-7, T47D, MDA-MB-231, vector-transfected and ERβ1-transfected MDA-MB-231 were treated with vehicle (DMSO) or 50 μM Res or TIMBD for 72 hours and MTT assays were performed. Percentage proliferation was determined by dividing the absorbance in Res- or TIMBD-treated cells by that in vehicle-treated cells X 100. Each experiment was performed in quadruplicate and data are expressed as percentage proliferation ± SEM relative to respective vehicle-treated controls.

b) LDH release assays were performed on breast epithelial and breast cancer cell lines as described in the Materials and Methods section. Percentage increase in LDH release was determined by dividing the difference of absorbances between Res-, TIMBD- and vehicle-treated cells to the difference of absorbances between total intracellular LDH and vehicle-treated cells X 100. Each experiment was performed in triplicate and data are expressed as percentage LDH release ± SEM relative to respective vehicle-treated controls (taken as 0%).

c–h) Breast epithelial and breast cancer cell lines were treated with vehicle (DMSO) or graded doses of TIMBD for up to 72 hours and LDH release assays were performed. Percentage increase in LDH release was determined as described above.

i) Breast epithelial and breast cancer cell lines were treated with vehicle (DMSO) or 50 μM Res or TIMBD for 48 hours and BrdU incorporation assays were performed. Each experiment was performed in triplicate and the data are expressed as percentage BrdU incorporation ± SEM.

(*) indicates a P value <0.05 compared to respective controls.

Fig. 2. TIMBD induces mRNA and protein expression levels of ERβ

a) MCF-7, T47D and MDA-MB-231 cells were treated with vehicle (DMSO) or 50 μ M Res or TIMBD for 24 hours. Total RNA was isolated and reverse transcribed to cDNA. Real-time quantitative PCR was performed to analyze the expression of cyclophilin and ERβ mRNA. Expression of ERβ mRNA was determined by dividing the number of cDNA molecules of ERβ by the number of cDNA molecules of cyclophilin, a housekeeping gene. Fold change was determined by dividing the expression of ERβ in the Res- or TIMBD-treated cells by the expression of ERβ in vehicle-treated cells (taken as 1). Each experiment was performed in quadruplicate and the data are expressed as fold change $\pm$ SEM relative to control.

b) MCF-10A and breast cancer cell lines were treated with vehicle (DMSO) or 50 μ M doses of Res or TIMBD for up to 72 hours. Proteins were isolated and western blot analyses were performed as described in the Materials and Methods section. Intensities of the bands were quantified and normalized to α -tubulin. Fold changes in ERβ protein expression (Mean $\pm$ SEM) in respective cell lines or transgene tagged ERβ1 protein expression (Mean $\pm$ SEM) in ERβ1-transfected MDA-MB-231 cells treated with Res or TIMBD compared to vehicle-treated controls of the same cell type were calculated from four individual experiments and the mean values are given at the top of each blot. Representative western blots are shown for each cell line mentioned above at the time point of maximal induction of ERβ in that cell line (MCF-10A = 24 hours; MDA-MB-231, vector-transfected and ERβ1-transfected MDA-MB-231 = 12 hours; MCF-7 and T47D = 48 hours). The vertical lines in the boxes indicate

that intervening lanes have been removed. Each individual western blot panel compares signals from protein samples loaded on the same gel.

c) MCF-7, T47D and MDA-MB-231 cell lines were treated with vehicle (DMSO) or 50 μ M TIMBD up to 72 hours and a time-course study was performed. Intensities of the bands were quantified and normalized to α -tubulin. Fold changes compared to vehicle-treated controls were calculated from four individual experiments. The bar graph represents fold change in ER β protein expression (Mean $\pm$ SEM) in respective cell lines treated with Res or TIMBD compared to vehicle-treated controls.

d) MDA-MB-231 cells were treated with vehicle or 25 to 100 μ M doses of TIMBD for 12 hours and a dose-response study was performed. Intensities of the bands were quantified and normalized to α -tubulin. Fold changes in ER β protein expression (Mean $\pm$ SEM) compared to vehicle-treated control were calculated from four individual experiments and the mean values are given at the top of each blot.

(*) indicates a P value <0.05 compared to respective controls.

Fig. 3. TIMBD inhibits mRNA and protein expression levels of ERα

a) MCF-7 and T47D cells were treated with vehicle (DMSO) or 50 μ M Res or TIMBD for 24 hours. Total RNA was isolated and reverse transcribed to cDNA. Real-time quantitative PCR was performed to analyze the expression of cyclophilin and ERα mRNA. Expression of ERα mRNA was determined as described in the Materials and Methods section. Each experiment was performed in quadruplicate and the data are expressed as fold change + SEM relative to control.

b) MCF-7 and T47D cells were treated with vehicle (DMSO) or 50 μ M Res or TIMBD for up to 72 hours. Proteins were isolated and western blot analyses were performed. Fold changes in ERα (Mean $\pm$ SEM) compared to vehicle-treated controls were calculated from four individual experiments and the mean values are given at the top of each blot.

Representative western blots are shown for MCF-7 and T47D cell lines after 48 hours of treatments, which is the time point of maximal inhibition of ERα in the breast cancer cell lines post-TIMBD treatment. The vertical lines in the boxes indicate that intervening lanes have been removed. Each individual western blot panel compares signals from protein samples loaded on the same gel.

c) MCF-7 and T47D cell lines were treated with vehicle (DMSO) or 50 μ M TIMBD for up to 72 hours and a time-course study was performed. Proteins were isolated and western blot analyses were performed. Intensities of bands were quantified and normalized to α -tubulin. Fold changes compared to vehicle-treated controls were calculated from four individual experiments. The bar graph represents fold change in ERα protein expression (Mean $\pm$ SEM) in respective cell lines treated with TIMBD compared to vehicle-treated controls.

d) T47D cells were treated with vehicle or 25 to 100 μ M doses of TIMBD for 48 hours and a dose-response study was performed. Proteins were isolated and western blot analyses were performed. Intensities of the bands were quantified and normalized to α -tubulin. Fold changes in ER α protein expression (Mean $\pm$ SEM) compared to vehicle-treated controls were calculated from four individual experiments and the mean values are given at the top of each blot.

(*) indicates a P value <0.05 compared to respective controls.

Fig. 4. TIMBD inhibits mRNA and protein expression levels of c-Myc and cyclin D1 in breast cancer cells

a) MCF-7, T47D and MDA-MB-231 cells were treated with vehicle (DMSO) or 50 μ M Res or TIMBD for 24 hours. Total RNA was isolated and reverse transcribed to cDNA. Real-time quantitative PCR was performed to analyze the expression of cyclophilin and c-Myc mRNA. mRNA expression levels of c-Myc and cyclophilin were determined as described in the Materials and Methods section. Each experiment was performed in quadruplicate and the data are expressed as fold change + SEM relative to control.

b) MCF-10A and breast cancer cell lines were treated with either vehicle (DMSO) or 50 μ M Res or TIMBD for up to 72 hours. Proteins were isolated and western blot analyses were performed. Intensities of the bands were quantified and normalized to α -tubulin. Fold changes in c-Myc protein expression (Mean $\pm$ SEM) treated with TIMBD compared to vehicle-treated controls were calculated from four individual experiments and the mean values are given at the top of each blot. The vertical lines in the boxes indicate that intervening lanes have been removed. Each individual western blot panel compares signals from protein samples loaded on the same gel.

c) MCF-7, T47D and MDA-MB-231 cells were treated with vehicle (DMSO) or 50 μ M Res or TIMBD for 24 hours. Total RNA was isolated and reverse transcribed to cDNA. Real-

time quantitative PCR was performed to analyze the expression of cyclophilin and cyclin D1 mRNA. Expression of cyclin D1 mRNA was determined as described in the Materials and Methods section. Each experiment was performed in quadruplicate and the data are expressed as fold change $\pm$ SEM relative to control.

d) MCF-10A and breast cancer cell lines were treated with either vehicle (DMSO) or 50 μ M Res or TIMBD for up to 72 hours. Proteins were isolated and western blot analyses were performed. Intensities of the bands were quantified and normalized to α -tubulin. Fold changes in cyclin D1 protein expression (Mean $\pm$ SEM) treated with TIMBD compared to vehicle-treated controls were calculated from four individual experiments and the mean values are given at the top of each blot. The vertical lines in the boxes indicate that intervening lanes have been removed. Each individual western blot panel compares signals from protein samples loaded on the same gel.

(*) indicates a P value <0.05 compared to respective controls.

Fig. 5. TIMBD induces protein expression levels of tumor suppressor genes p53 and p21 in MCF-7 breast cancer cells

a) MCF-7 breast cancer cells were treated with vehicle (DMSO) or 50 μ M Res or TIMBD. Proteins were isolated and western blot analyses were performed. Intensities of the bands were quantified and normalized to α -tubulin. Fold changes in p53 and p21 protein expression (Mean $\pm$ SEM) treated with TIMBD compared to vehicle-treated controls were calculated from four individual experiments and the mean values are given at the top of each blot. The vertical lines in the boxes indicate that intervening lanes have been removed. Each individual western blot panel compares signals from protein samples loaded on the same gel.

b) MCF-7 cells were treated with either vehicle (DMSO) or 50 μ M Res or TIMBD up to 24 hours and a time-course study was performed. Proteins were isolated and western blot analyses were performed. Intensities of the bands were quantified and normalized to α -tubulin. The bar graph represents fold change in p53 or p21 protein expression levels (Mean $\pm$ SEM) in respective cell lines treated with Res or TIMBD compared to vehicle-treated controls.

(*) indicates a P value <0.05 compared to respective controls.

Fig. 6a

Fig. 6b

TIMBD

Fig. 6. TIMBD inhibits protein expression c-Myc in an ER-dependent fashion in breast cancer cells

a) MCF-10A, ER β 1-transfected MDA-MB-231, MCF-7 and T47D cells were transfected with either 1 nmol/l of scrambled small interfering RNA or siER β for 48 hours, and subsequently treated with 50 μ M TIMBD for 12 hours (ER β 1-transfected MDA-MB-231), 24 hours (MCF-10A) or 48 hours (MCF-7 and T47D). The treatment time points are based on maximal induction time of ER β for respective cell lines following TIMBD treatment. Proteins were isolated and western blot analyses were performed. Intensities of the bands were quantified and normalized to α -tubulin. Fold changes in ER β or c-Myc protein expression (Mean $\pm$ SEM) treated with scrambled, siER β or TIMBD compared to vehicle-controls were calculated from four individual experiments and the mean values are given at the top of each blot.

b) Binding of Resveratrol analog TIMBD into the active site of ER β , as assessed by computer modeling studies.

(*) indicates a P value <0.05 compared to respective controls.

Fig. 7. TIMBD partially inhibits proliferation of MDA-MB-231-ER β transfected cell line in an ER β -dependent fashion

a) MDA-MB-231-ER β cells were seeded in 6-well plates and were transfected with siRNA for ER β followed by treatment with TIMBD or vehicle for 12 hours. After treatment, cells were incubated for an additional 8 days in fresh complete medium. The colonies obtained were fixed in methanol and stained with crystal violet solution. Three replicates were performed for each treatment group and representative pictures for each treatment group are shown.

b) MDA-MB-231-ER β transfected cells were seeded into 24-well plates and mammosphere assays were performed. Cells were grown in serum-free medium supplemented with growth factors as mentioned in Materials and Methods section. Cells were treated with TIMBD or vehicle control and the mammospheres formed were viewed under the microscope and photographed [25, 27, 30]. Three replicate wells from a 24-well plate were used for each experimental condition and representative pictures for each treatment group are shown. Western blot for ER β after siRNA transfection is also presented to demonstrate that ER β protein expression was decreased following siER β transfection.

Fig. 8a.

Fig. 8b.

Fig. 8. TIMBD is equally effective in inhibiting the proliferation of MDA-MB-231 and ER α -transfected MDA-MB-231 cells

a) MDA-MB-231 cells were transiently transfected with ER α and empty-vector plasmids using Lipofectamine 2000 transfection reagent for 48 hours in 6-well plates. Following transfection, cells were re-plated in 96-well plates and treated with 50 μ M TIMBD or vehicle control for additional 48 hours. MTT assays were performed as described in Materials and Methods section. Three replicate wells were used for each experimental condition. Western blot for ER α expression in MDA-MB-231 cells following transfection is included to demonstrate that ER α protein expression was increased following ER α transfection.

b) MDA-MB-231-ER α or vector-transfected cells were seeded in 6-well plates followed by treatment with TIMBD or vehicle for 48 hours. After treatment, cells were incubated for an additional 8 days in a complete medium. The colonies obtained were fixed in methanol and stained with crystal violet solution. Three replicates were performed for each treatment group and representative pictures are shown. Resulting colonies were counted and the bar graph represents the % colonies formed (Mean $\pm$ SEM) relative to respective vehicle-treated controls of the same cell type.

(*) indicates a P value <0.05 compared to respective controls.

Table 1
Ratio of ER β to ER α protein expressions in MCF-7 and T47D cell lines treated with TIMBD

MCF-7 and T47D breast cancer cell lines were treated with vehicle (DMSO) or 50 μ M Res or TIMBD for 48 hours, which is the time point of maximal induction of ER β and maximal inhibition of ER α in these cell lines. Proteins were isolated and western blot analyses were performed. Intensities of the bands were quantified and normalized to α -tubulin. Fold changes in ER β or ER α protein expression (Mean $\pm$ SEM) treated with Res or TIMBD compared to vehicle-treated controls were calculated from four individual experiments and ratios of ER β :ER α were determined.

Cell line	Treatment	Fold ER β	Fold ER α	Ratio (ER β /ER α)
MCF-7	Res	1	0.2	5
	TIMBD	2.9	0.5	5.8
T47D	Res	1.9	0.9	2.1
	TIMBD	2.7	0.2	13.5

Table 2

Docking results and consensus scores of Resveratrol analog TIMBD

Chemical	Binding Energy (Kcal/mole)	Hydrogen bonding residues	Bond distance (Å)	No. of Hydrogen bonds
Resveratrol	-8.2	GLY472 GLU305 ARG346	2.0 2.2 and 2.3 2.3	4
TIMBD	-8.3	GLY472, HIS475	2.0, 2.1	2