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Potent Inhibition of Breast Cancer by Bis-Indole-Derived Nuclear Receptor 4A1 (NR4A1) Antagonists

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Abstract

Background: Nuclear receptor 4A1 (NR4A1) is overexpressed in mammary tumors, and the methylene-substituted bis-indole derivative 1,1-bis(3'-indolyl)-1-(*p*-hydroxyphenyl)methane (DIM-C-pPhOH) acts as an NR4A1 antagonist (inverse agonist) and inhibits NR4A1-regulated pro-oncogenic pathways/genes in breast and other cancer cells.

Methods: Butressed analogs of DIM-C-pPhOH were synthesized by condensation of the substituted *p*-hydroxybenzaldehydes with indole. Breast cancer cell growth, survival and migration assays were carried out by cell counting, Annexin V staining and Boyden chamber assays, respectively. Changes in RNA and protein expression were determined by RT-PCR and western blots, respectively. Analysis of RNAseq results was carried out using Ingenuity Pathway Analysis, and *in vivo* potencies of NR4A1 antagonists were determined in athymic nude mice bearing MDA-MB-231 cells in an orthotop model.

Results: Ingenuity Pathway analysis of common genes modulated by NR4A1 knockdown or treatment with DIM-C-pPhOH showed that changes in gene expression were consistent with the

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Human and Animal Rights:

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observed decreased functional responses, namely inhibition of growth and migration and increased apoptosis. DIM-C-pPhOH is rapidly metabolized and the effects and potencies of buttressed analogs of DIM-C-pPhOH which contain one or two substituents *ortho* to the hydroxyl groups were investigated using NR4A1-regulated gene/gene products as endpoints. The buttressed analogs were more potent than DIM-C-pPhOH in both *in vitro* assays and as inhibitors of mammary tumor growth. Moreover, using 1,1-bis(3'-indolyl)-1-(3-chloro-4-hydroxy-5-methoxyphenyl)methane (DIM-C-pPhOH-3-Cl-5-OCH₃) significant tumor growth inhibition was observed at doses as low as 2 mg/kg/d which was at least an order of magnitude more potent than DIM-C-pPhOH.

Conclusions: These buttressed analogs represent a more potent set of second generation NR4A1 antagonists as inhibitors of breast cancer.

Keywords

NR4A1; bis-indole ligands; antagonists; anticancer activities

BACKGROUND

The 48 members of the nuclear receptor (NR) superfamily of transcription factors play essential roles in maintain cellular homeostasis and are also potential druggable targets for many diseases including cancer [1,2]. Pharmaceutical agents that directly target NRs are often selective receptor modulators which bind the receptor and exhibit tissue-specific receptor agonist or antagonist activities [2]. For example, tamoxifen is a widely used selective estrogen receptor modulator (SERM) for treating breast cancer and tamoxifen exhibits ER antagonist activity against breast cancer but is an ER agonist in the uterus [rev. in [3,4]. Differential expression of some NRs in cancer vs. noncancer tissues has been observed and these receptors may also be prognostic factors for drug-resistance, patient survival, and disease recurrence [5-11]. Muscat and coworkers [7] investigated the relative expression of NRs in breast tumors and normal breast and identified 41 NRs in breast cancers and 33 of these receptors exhibited differential expression in tumor vs. non-tumor tissues. Twenty-six NRs were higher in normal breast vs. ER-positive and ER-negative breast cancer and only seven NRs were more highly expressed in tumors. Two of the 3 NRs overexpressed in both ER-positive and ER-negative breast tumors were members of the NR4A subfamily, namely NR4A1 (Nur77, TR3) and NR4A3 (Nor1) [7]. Subsequent studies on the expression, prognostic value, and functions of NR4A1 in breast cancer are contradictory and functional studies report that NR4A1 exhibits both pro- and anticarcinogenic activities [11-16].

Research in this laboratory has focused on a series of methylene-substituted bis-indole-derived compounds (C-DIMs) as NR4A1 ligands which exhibit tissue-specific NR4A1 agonist or antagonist activities and are selective NR4A1 modulators [8,14-22]. In breast cancer and other solid tumors NR4A1, acts as a pro-oncogenic factor regulating pathways/ genes such as β 1-integrin and TXNDC5 associated with cell proliferation, survival and migration/invasion and NR4A1 ligands typified by 1,1-bis(3'-indolyl)-1-(*p*-hydroxyphenyl)methane (DIM-C-pPhOH, CDIM8) act as antagonists [8,14-20]. Previous studies in breast, colon, kidney, pancreatic and lung cancer cell lines and rhabdomyosarcoma

cells show that both knockdown of NR4A1 (siNR4A1) or treatment with DIM-C-pPhOH decreased cell growth and migration, induced apoptosis, and regulated expression of genes associated with these responses [8,14-21]. Although DIM-C-pPhOH inhibits tumor growth in xenograft models (30 mg/kg/d) [17,21,8], this compound is rapidly metabolized [23]. Therefore, we synthesized several buttressed analogs of DIM-C-pPhOH containing at least one or two substituents *ortho* (3'- or 3',5'-) to the hydroxyl groups since it has previously been reported that this substitution pattern hinders metabolism (conjugation) of phenolic compounds [24]. The *in vitro* results clearly demonstrate the enhanced potency of the buttressed analogs compared to DIM-C-pPhOH and the IC₅₀ for breast tumor growth inhibition by one of the buttressed analog 1,1-bis(3'-indolyl)-1-(3-chloro-4-hydroxy-5-methoxyphenyl)methane (DIM-C-pPhOH-3-Cl-5-OCH₃) was approximately 2 mg/kg/d and greater than 10-fold more potent than DIM-C-pPhOH.

MATERIALS AND METHODS

Cell lines, antibodies and chemicals.

MDA-MB-231 and SKBR3 human breast cancer cell lines were purchased from American Type Culture Collection (Manassas, VA). MCF10A cells were kindly provided by Dr. Weston Porter, Texas A&M University. Cells were maintained 37°C in the presence of 5% CO₂ in Dulbecco's modified Eagle's medium/Ham's F-12 medium with 10% fetal bovine serum with antibiotic. β-Actin antibody and Dulbecco's Modified Eagle's Medium were purchased from Sigma-Aldrich (St. Louis, MO). Sp1 antibody was purchased from Millipore (Temecula, CA); bcl2, CHOP and epidermal growth factor receptor (EGFR) antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA). Cleaved caspase 3, cleaved poly ADP ribose polymerase (c-PARP), phospho mTOR, mTOR, phospho S6RP, S6RP, phospho 4EBP1, 4EBP1, XBP1-S, SERPINB5, GADD45α, and p21 antibodies were purchased from Cell Signaling Technologies (Danvers, MA). TXNDC5 and IDH1 antibodies were purchased from Genetex (Irvine, CA). Apoptotic, Necrotic, and Healthy Cells Quantification Kit was purchased from Biotium (Hayward, CA). Cells were visualized under an EVOS fl, fluorescence microscope, from Advanced Microscopy Group using a multiband filter set for FITC, rhodamine, and DAPI. The C-DIM compounds were prepared as previously described by the condensation of indole with substituted benzaldehydes [9-11], and Supplemental Table S1 summarizes the substituted benzaldehydes purchased from Sigma Aldrich that were used to synthesize the buttressed analogs.

Cell proliferation assay.

MDA-MB-231 and SKBR3 breast cancer cells and MCF10A cells (1.0×10^5 per well) were plated in 12-well plates and allowed to attach for 24 h, and cells were treated with various C-DIM analogs (dimethyl sulfoxide, DMSO, as empty vehicle) for 18-24 h and effects on cell proliferation were determined as described [14]. **Annexin V staining.** MDA-MB-231 and SKBR3 breast cancer cells (1.0×10^5 per well) were seeded in 2-well Nunc Lab-Tek chambered B#1.0 Borosilicate coverglass slides from Thermo Scientific (Waltham, MA) and were allowed to attach for 24 h and treatment-related effects on Annexin V staining were determined as described [14].

Boyden chamber assay.

MDA-MB-231 and SKBR3 breast cancer cells (3.0×10^5 per well) were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed to attach for 24 h. and treatment-related effects on Annexin V staining were determined as described [14,15].

RNA sequencing analysis.

SKBR3 cells were transfected with siNR4A1 (72 h) or treated with DMSO or DIM-C-pPhOH for 24 h, and mRNA was isolated for RNAseq determining treatment-related genes vs. control (DMSO). The RNAseq reads were mapped with the STAR aligner using the default parameters [25] and human genome assembly GRCh38. Differentially expressed genes were determined using R package EdgeR [26]. Genes with the fold change ≥ 1.5 or ≤ -1.5 and p value < 0.05 were selected to further analyze overlapping genes between siTR3/siDMSO vs. DIM8 treated group using R software. The RNAseq was determined in the Texas A&M University Genomics and Bioinformatics core facility.

RT PCR.

RNA was isolated using Zymo Research Quick-RNA MiniPrep kit (Irvine, CA). Quantification of mRNA (SERPINB5, GADD45 α , p21) was performed using Bio-Rad iTaq Universal SYBER Green 1-Step Kit (Richmond, CA) using the manufacturer's protocol with real-time PCR. TATA Binding Protein (TBP) mRNA was used as a control to determine relative mRNA expression. Primers used for RT-PCR include (1) SERPINB5: forward – TCC CTG CTC CTT CAT TCT C, reverse – GCC TCT GGA TTC TGG CTC T; (2) p21: forward – TGA GCC GCG ACT GTG ATG, reverse – GTC TCG GTG ACA AAG TCG AAG TT; (3) GADD45A: forward – CGT TTT GCT GCG AGA ACG AC, reverse – GAA CCC ATT GAT CCA TGT AG

Western blot analysis.

The breast cancer cell lines and MCF10A cells (3.0×10^5 per well) were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium in 6-well plates. Cells were allowed to attach for 24 h after they were seeded and subsequently treated with varying concentrations of C-DIM analogs for 24 h and western blots were determined as described [14-16].

TNBC orthotopic xenograft model.

Female BALB/c nude mice (6-8 weeks old) were obtained (Charles River Laboratory, Wilmington, MA) and maintained under specific pathogen-free conditions, housed in isolated vented cages, and allowed to acclimate for one week with standard chow diet. The animals were housed at Texas A&M University Laboratory Animal Resources and Research facility in accordance with the standards of the Guide for the Care and Use of Laboratory Animals and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). MDA-MB-231 cells (1×10^6 cells) were used in the athymic nude mouse xenograft studies as previously described [14-16]. Tumor weights were determined after necropsy and the tumor size was measured using Vernier calipers (everyday). The tumor

volume was estimated by the formula: tumor volume (mm^3) = $(L \times W^2) \times \frac{1}{2}$, where L is the length and W is the width of the tumor. Tumor lysates were obtained and analyzed for protein expression by western blots.

Statistical analysis.

Statistical significance of differences between the treatment groups was determined by student's *t* test. The results are expressed as means with error bars representing 95% confidence intervals for 3 experiments for each group unless otherwise indicated, and a *P* value less than 0.05 was considered statistically significant. All statistical tests were 2-sided.

RESULTS

NR4A1 is a pro-oncogenic factor overexpressed in breast tumors that can be targeted by C-DIM/NR4A1 antagonists and we have previously reported that NR4A1 protein is highly expressed and functional in SKBR3, MDA-MB-231 and other breast cancer cell lines [7,11,14,15]. This study initially focused on identification of NR4A1-regulated genes by RNAseq and then on identifying more potent NR4A1 antagonists using NR4A1 responsive genes to determine structure-activity relationships. SKBR3 cells were either transfected with siNR4A1 (knockdown) or treated with 20 μM of the NR4A1 antagonist DIM-C-pPhOH and knockdown efficiencies were > 80% as previously described [14] RNAseq analysis shows that compared to control (DMSO) cells, altered expression of 981 and 1823 genes was observed, respectively (Fig. 1A). Moreover, both treatments induced 137 and decreased expression of 237 genes in common, including induction of GADD45A, SERPINB5 (maspin) and CDKNA (p21). Causal IPA analysis of the changes in gene expression by siNR4A1 and DIM-C-pPhOH predicted decreased cell proliferation ($p = .000355$; $z\text{-score} = 3.94$), migration of cancer cells ($p = 0.00613$; $z\text{-score} = 2.78$), and induction of apoptosis ($p = 0.000527$; $z\text{-score} = 2.90$) (Fig. 1B). We also examined high and low expression of NR4A1 in human breast tumors; although there was a trend for decreased survival associated with high expression of NR4A1, the differences (high vs. low) were not significant (Fig. 1C). NR4A1 expression in tumors from these patients was observed in early to late stage cancers with minimal variability (Fig. 1D).

DIM-C-pPhOH is a prototypical NR4A1 ligand that exhibits antagonist activities in breast and other cancer cell lines; however, pharmacokinetic studies indicate that this compound exhibits low serum levels and is rapidly metabolized [23]. Therefore, we have synthesized a series of buttressed analogs of DIM-C-pPhOH. These compounds (Fig. 2A) contain at least one 3' or 5' substituent in order to decrease the rate of metabolism via conjugation of the hydroxyl group [24]. Like DIM-C-pPhOH, all of the buttressed analogs inhibited transactivation (luciferase activity) in SKBR3 cells transfected with chimeric GAL4-NR4A1 and a reporter gene (UAS-luc) containing 5 GAL4 response element (Fig. 2A). The results of this assay did not distinguish between the relative potencies of DIM-C-pPhOH and the buttressed analogs but demonstrated that all of these compounds antagonized NR4A1-dependent transactivation. DIM-C-pPhOH decreased expression of TXNDC5 and β 1-integrin proteins in MDA-MB-231 and SKBR3 breast cancer cells (Fig. 2B) as previously described in breast and other cancer cell lines at doses 15 or 10 μM , respectively

[17,14,15,21,8]. For Comparative screening we used a lower concentration (5 μM) of the buttressed analogs and observed decreased expression of $\beta 1$ -integrin and TXNDC5 in MDA-MB-231 (Fig. 2C) and SKBR3 (Fig. 2D) breast cancer cells, suggesting that these compounds are more potent than DIM-C-pPhOH.

MDA-MB-231 and SKBR3 cells express relative high levels of NR4A1 and were responsive to DIM-C-pPhOH and were used as representative cell lines in this study [14-16]. Results of the RNAseq studies identified several genes that are induced in MDA-MB-231 cells treated with DIM-C-pPhOH or transfected with siNR4A1 and this includes SERPINB5 (maspin), GADD45 α and p21. Figure 3A illustrates induction of these proteins by DIM-C-pPhOH (20 μM) in MDA-MB-231 and SKBR3 cells and induction was observed after treatment with 15-20 μM DIM-C-pPhOH. In contrast, 5 μM concentrations of all the buttressed analogs induced p21 and SERPINB5 protein in MDA-MB-231 (Fig. 3B) and SKBR3 (Fig. 3C) cells and GADD45 α in SKBR3 cells, whereas only the 3,5-dibromo-, 3-chloro-5-methoxy- and 3-chloro-substituted analogs were potent inducers in MDA-MB-231 cells. We used the latter three compounds as model "second generation" NR4A1 antagonists, namely 1,1-bis(3'-indolyl)-1-(3-chloro-4-hydroxyphenyl)methane (3-Cl), 1,1-bis(3'-indolyl)-1-(3,5-dibromo-4-hydroxyphenyl)methane (3,5-Br₂), and 1,1-bis(3'-indolyl)-1-(3-chloro-4-hydroxy-5-methoxyphenyl)methane (3-Cl-5-OCH₃). In MDA-MB-231 (Fig. 3D) and SKBR3 (Fig. 3E) cells, 1-500 nM concentrations induced SERPINB5, GADD45 α and p21 protein levels, demonstrating their increased potency as inducers of these gene products compared to DIM-C-pPhOH which was active in the 2.5-10 μM range. Supplemental Figure S1 illustrates the effects of these NR4A1 antagonists on genes associated with cell proliferation and survival (Suppl. Fig. S1A), mTOR signaling (Suppl. Fig. S1B), and $\beta 1$ -integrin/TXNDC5 and stress genes (Suppl. Fig. S1C) in MDA-MB-231 and SKBR3 cells. As previously observed in other cancer cells treated with DIM-C-pPhOH, the second generation NR4A1 antagonists induced or repressed expression of these proteins at concentrations ranging from 2-5 μM , whereas these same effects of DIM-C-pPhOH were observed at higher concentrations (15-20 μM) [17,14,21,8].

The relative potencies of the 3-Cl, 3,5-Br₂, and 3-Cl-5-OCH₃ analogs of DIM-C-pPhOH were determined as inducers of SERPINB5, GADD45 α and p21 mRNA levels in MDA-MB-231 (Fig. 4A) and SKBR3 (Fig. 4B) cells. EC₅₀ values for the induction responses (Table 1) were variable for different genes (low μM to high nM) and the most potent response was observed for induction of p21 mRNA by 3-Cl-5-OCH₃ in MDA-MB-231 cells (EC₅₀=0.19 μM). We also investigated the concentration-dependent effects of these compounds on the proliferation (24 h) of MDA-MB-231 and SKBR3 cells (Fig. 5A), induction of Annexin V staining (apoptosis) (Fig. 5B), and inhibition of cell migration (Fig. 5C). In contrast DIM-C-pPhOH and the buttressed analogs did not affect growth or induce PARP cleavage (apoptosis) in non-transformed MCF10A cells and NR4A1 levels were also low in this cell line (Supplemental Figure 2). EC₅₀ values for the functional responses (inhibition of cell growth and migration, induction of apoptosis) varied from 2.11-6.46 μM . Whereas EC₅₀ values for induction of gene expression varied from 0.19-4.97 μM (Table 1).

The dose of DIM-C-pPhOH that inhibits (30-60%) tumor growth in mouse xenograft models was 15-30 mg/kg/d [17,21,8]. In this study, we initially treated athymic nude mice bearing

MDA-MB-231 cells (orthotopic) with 3-Cl, 3,5-Br₂ and 3-Cl-5-OCH₃ at doses of 10 (Fig. 6A) and 5 (Fig. 6B) mg/kg/d. All compounds were potent inhibitors of tumor volume and weight. Supplemental Figure 3 illustrates the effects of these compounds on expression of diagnostic NR4A1-regulated gene products in tumors from untreated and treated (10 mg/kg/d) mice, and these results complement the effects of these compounds observed in *in vitro* studies (Figs. 2 and 3, Suppl. Fig. S1). The 3-Cl-5-OCH₃ compound was then tested at doses of 2, 1, 0.5 and 0.1 mg/kg/d, and significant inhibition of tumor volume (Fig. 6C) and weight (Fig. 6D) was observed only at the 2 mg/kg/d dose changes in body or organ weights were not observed in any of the treatment groups. Thus, the EC₅₀ for 3-Cl-5-OCH₃ is approximately 2 mg/kg/d which is markedly much lower than observed for DIM-C-pPhOH (30 mg/kg/d) and demonstrates that the buttressing effect successfully enhanced both the cell culture and *in vivo* potencies of the second generation C-DIM/NR4A1 antagonists.

DISCUSSION

Early stage ER-positive breast cancers respond to hormonal therapy which can include treatment with antiestrogens and aromatase inhibitors where these compounds specifically target estrogen signaling pathways [27,28]. In contrast, drugs that target NR4A1 in breast and other cancers inhibit multiple pro-oncogenic NR4A1-regulated genes that play a role in cancer cell growth, survival and migration/invasion [14-16]. These pathways have been extensively investigated in breast cancer where NR4A1 agonists inhibit mTOR signaling, include ROS and endoplasmic reticulum stress, induce apoptosis, inhibit growth promoting genes including EGFR and migration genes such as β 1- and β 3-integrins [8,14-16,29,30] and many of these responses have been observed in other cancer lines. The NR4A1 antagonist DIM-C-pPhOH also inhibits TGF β -induced invasion of breast cancer cells (which is accomplished by enhancing p38 α -dependent NR4A1 phosphorylation and nuclear export which activates proteasome-mediated degradation of SMAD7 [16]. DIM-C-pPhOH inhibits phosphorylation of NR4A1 and its subsequent nuclear export, thereby inhibiting this pathway and stabilizing inhibitory SMAD7 levels [16].

In this study, we initially carried out RNAseq analysis of genes that are affected by DIM-C-pPhOH or knockdown of NR4A1 to investigate and analyze genomic pathways regulated by NR4A1. Causal IPA integrates changes in expression of multiple genes to determine a z-score which represents a statistical match between observed changes in gene expression with the expected direction of a specific pathway. A z-score >2 or <-2 is considered to be significant and IPA analysis of changes in gene expression associated with cell proliferation (decreased), induction of apoptosis (increased) and cell migration (decreased) were -3.94, 2.90 and -2.78, respectively. These results confirm previous studies on the pro-oncogenic functions of NR4A1 observed in breast and other cancers [8,14-22].

Our second major objective was to investigate the relative *in vitro* and *in vivo* potencies of several buttressed analogs of DIM-C-pPhOH which should exhibit increased potency due to decreased metabolism (conjugation) because of a "buttressing" effect [24]. Our initial screen using ligand-dependent NR4A1-mediated downregulation of β 1-integrin and TXNDC5 proteins (Fig. 2) showed that the buttressed analogs were more potent than DIM-C-pPhOH and similar results were observed for induction of NR4A1-responsive genes p21,

SERPINB5 and GADD45 α (Fig. 3). Induction of these gene products in MDA-MB-231 and SKBR3 cells by three buttressed analogs, namely DIM-C-pPhOH-3-Cl (3-Cl), DIM-C-pPhOH-3,5-Br₂ (3,5-Br₂), and DIM-C-pPhOH-3-Cl-5-OCH₃ (3-Cl-5-OCH₃), was variable and gene product-dependent but in the 10-50 nM range for most responses (Figs. 3D and 3E). In contrast, DIM-C-pPhOH induced these same response at much higher concentrations (2.5-10 μ M) (Fig. 3A). We also quantitated the effects of these buttressed analogs against functional responses (inhibition of growth and migration and induction of apoptosis) and gene expression responses. IC₅₀ values for induction of SERPINB5, GADD45 α and p21 varied from 0.19-4.97 μ M and IC₅₀'s for functional responses ranged from 2.11-6.46 μ M (Table 1). Previous xenograft studies showed that DIM-C-pPhOH inhibited 40-60% of tumor growth at doses of 30 mg/kg/d [17,21,8] and it was assumed that the buttressed analogs would have increased potency due to decreased metabolism via conjugation of the hydroxyl group. Our initial screening showed that 3-Cl, 3,5-Br₂ and 3-Cl-5-OCH₃ inhibited tumor growth at doses of 10 and 5 mg/kg/d. Lower doses of 3-Cl-5-OCH₃ showed that an IC₅₀ value was approximately 2 mg/kg/d with only minimal tumor growth inhibition observed at a dose of 1 mg/kg/d (Fig. 6), thus demonstrating significantly increased *in vivo* potency for the buttressed analogs of DIM-C-pPhOH.

CONCLUSIONS

In summary, our previous results with DIM-C-pPhOH and studies with the buttressed analogs show that these compounds are NR4A1 antagonists that inhibit cell growth and migration and induce apoptosis in MDA-MB-231 and SKBR3 cells but not in non-transformed MCF10A cells (Supplemental Fig. 2). IPA analysis of common genes affected by DIM-C-pPhOH and siNR4A1 confirm results of functional assays, indicating that NR4A1 is pro-oncogenic in breast cancer. The new buttressed analogs are active *in vitro* in the low μ M range and the significant inhibition of tumor growth in a mouse xenograft model using MDA-MB-231 cells was observed at doses as low as 2 mg/kg/d. In contrast the compounds did not affect organ or body weights and in a previous study treatment of mice (on high fat diet) with 25 mg/kg/d of the 3-Cl-5-OCH₃ buttressed analog for 8 weeks did not induce and body or organ weight changes (22). These results demonstrate the enhanced potency of the buttressed analogs for *in vivo* studies and these compounds are being further developed for clinical applications in treating breast and other solid tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

C-DIMs
methylene-substituted DIMs

DIM-C-pPhOH1,1-bis(3'-indolyl)-1-(*p*-hydroxyphenyl)methane**DIM-C-pPhOh-3-Cl-5-OCH₃**

1,1-bis(3'-indolyl)-1-(3-chloro-4-hydroxy-5-methoxyphenyl)methane

DMSO

dimethyl sulfoxide

EGFR

epidermal growth factor receptor

NR4A1

nuclear receptor 4A1

SERMs

selective estrogen receptor modulators

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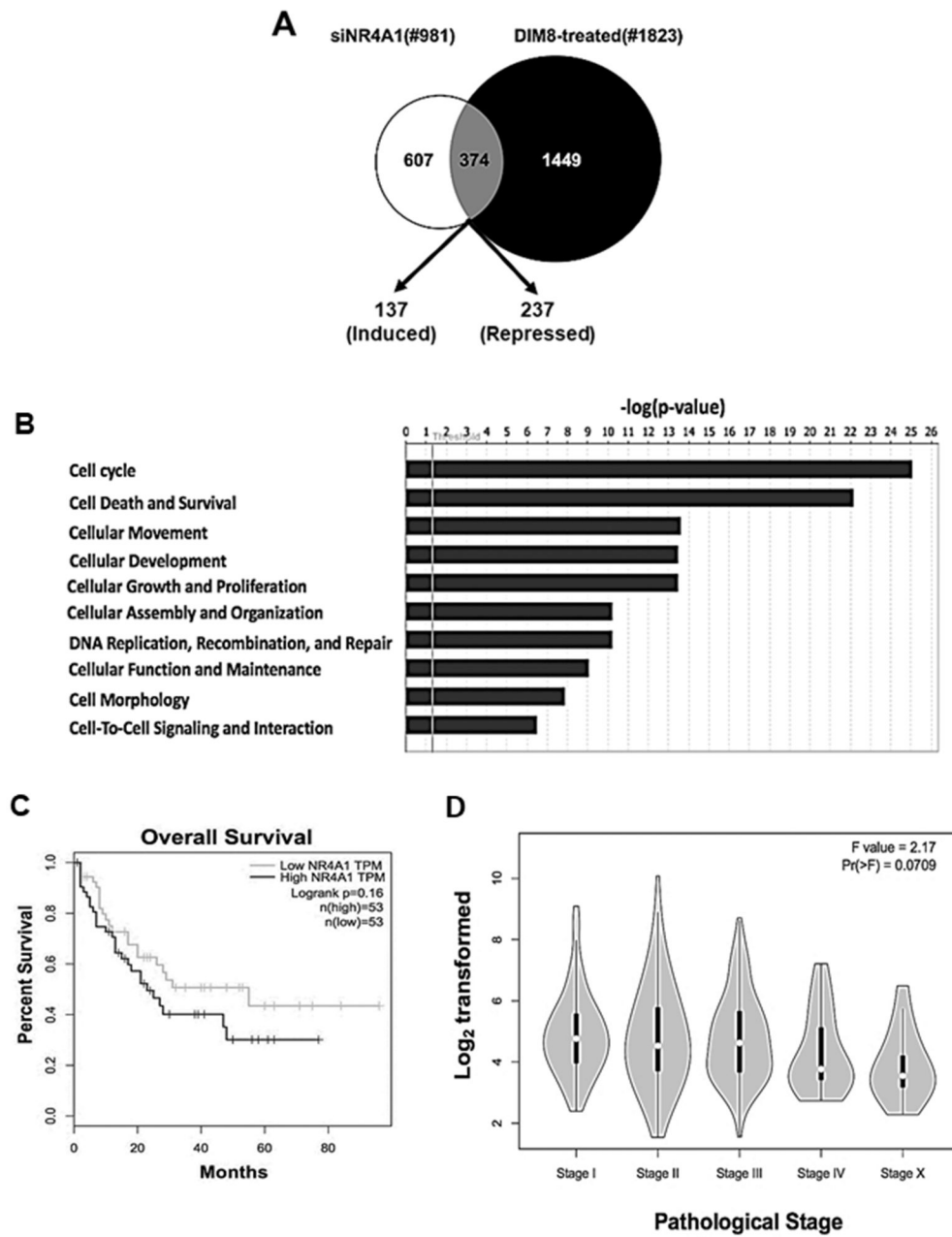
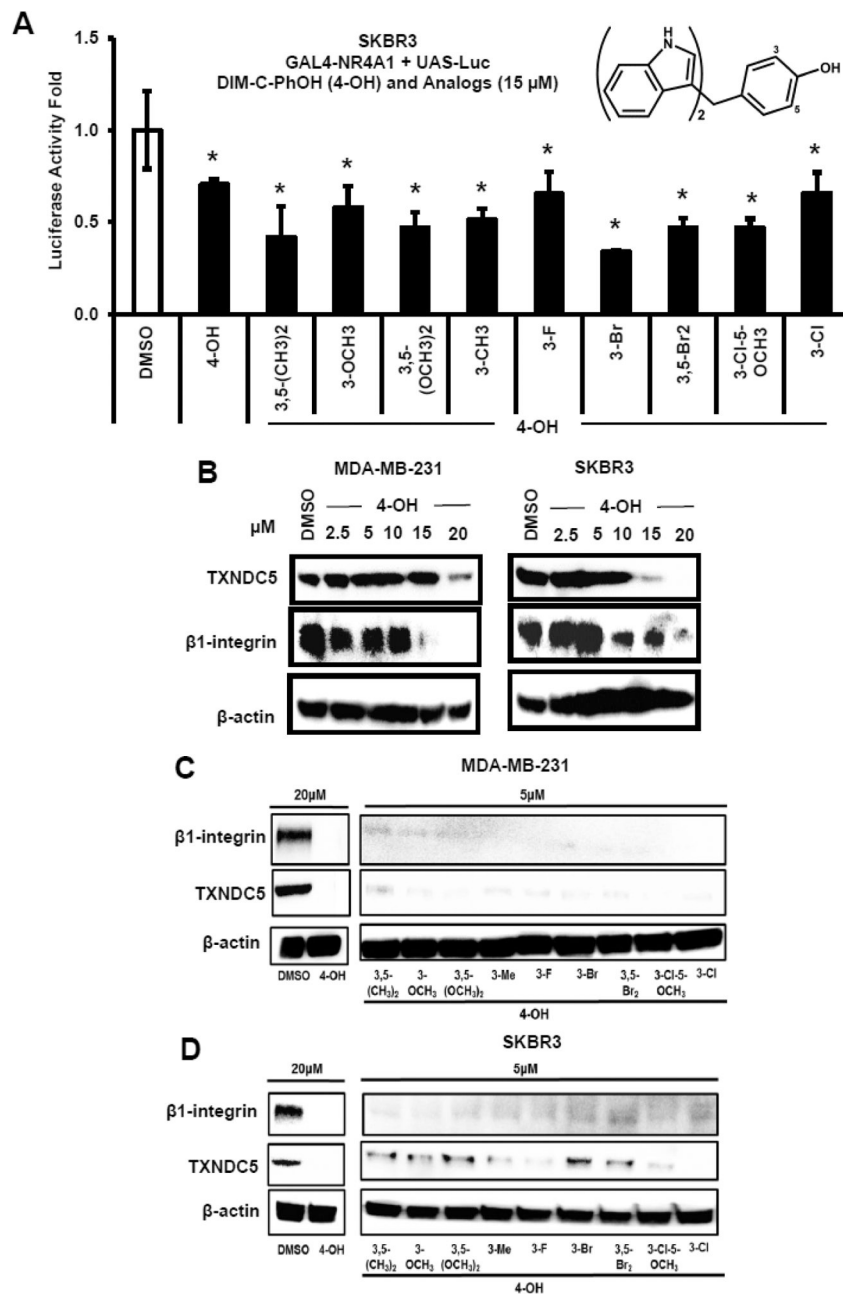
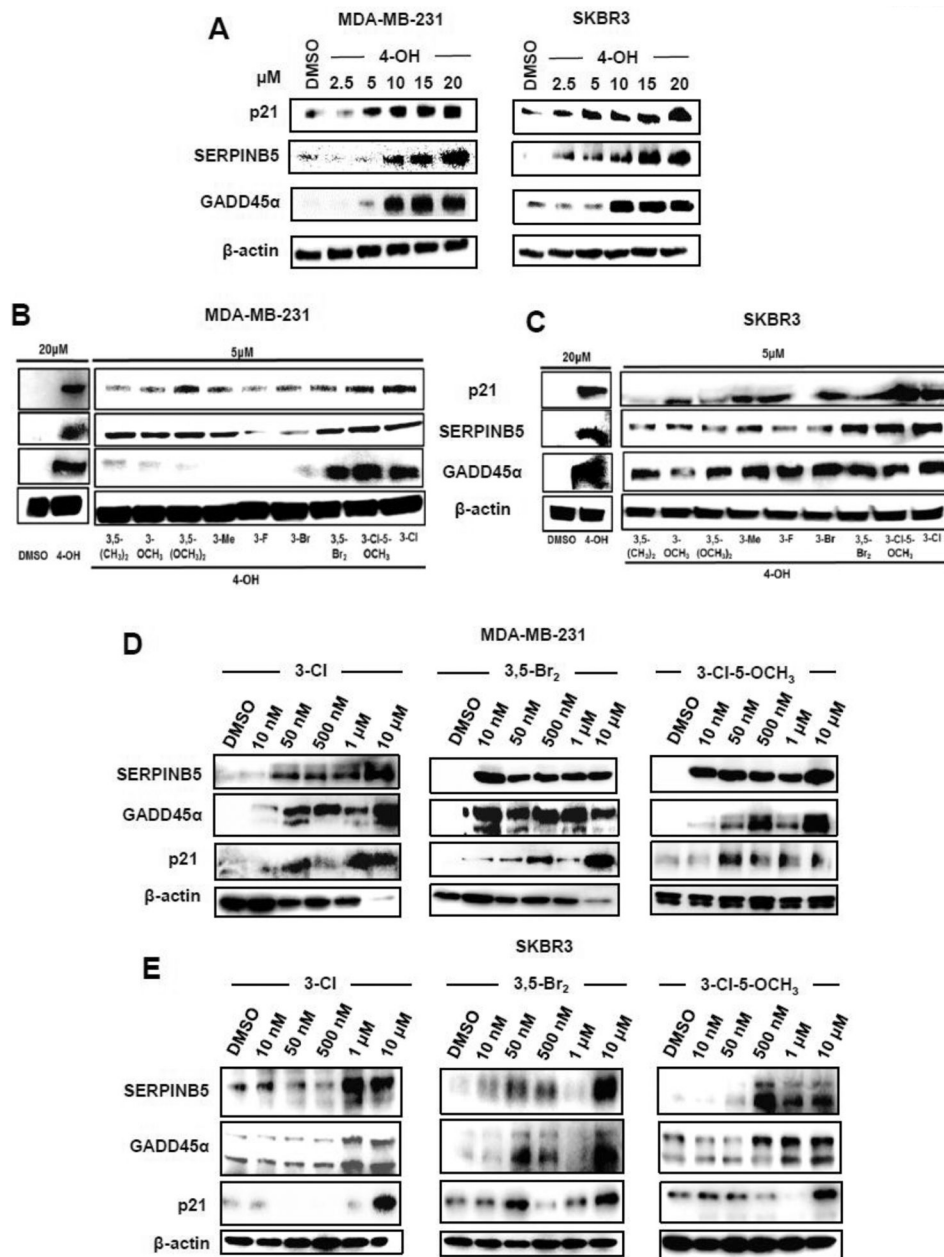


Figure 1.

NR4A1-regulated gene expression in breast tumors. MDA-MB-231 cells were transfected with iNR4A1 or treated with 20 μ M DIM-C-pPhOH (CDIM8) for 24 h, and mRNA from each treatment was analyzed by RNAseq (A) and normalized data was analyzed by IPA (B). The NCBI database was analyzed for expression of NR4A1 mRNA and correlated with overall patient survival (C) and NR4A1 levels (y-axis) expressed in tumors assigned as Stage I-IV and metastasis (Stage X) (D).

**Figure 2.**

Screening for buttressed analogs of DIM-C-pPhOH as NR4A1 antagonists. (A) SKBR3 cells were transfected with GAL4-NR4A1 and UAS-luc, treated with DIM-C-pPhOH and 9 buttressed analogs, and luciferase activity was determined as outlined in the Materials and Methods. (B) MDA-MB-231 and SKBR3 cells were treated with different concentrations of DIM-C-pPhOH (4-OH) for 24 h, and whole cell lysates were analyzed by western blots for downregulation of β 1-integrin and TXNDC5. MDA-MBA-231 (C) and SKBR3 (D) cells were treated with 20 μ M DIM-C-pPhOH (4-OH) and 5 μ M concentrations of 9 buttressed analogs for 24 h, and whole cell lysates were analyzed by western blots for downregulation of β 1-integrin and TXNDC5.

**Figure 3.**

Induction of NR4A1-dependent genes by DIM-C-pPhOH and buttressed analogs in breast cancer cells. (A) MDA-MB-231 and SKBR3 cells were treated with different concentrations of DIM-C-pPhOH (4-OH) for 24 h, and whole cell lysates were analyzed by western blots for induced expression of p21, SERPINB5 and GADD45α. MDA-MB-231 (B) and SKBR3 (C) cells were treated with 20 μM DIM-C-pPhOH (4-OH) or 5 μM of 9 buttressed analogs of DIM-C-pPhOH for 24 h, and whole cell lysates were analyzed by western blots for induction of p21, SERPINB5 and GADD45α. MDA-MB-231 (D) and SKBR3 (E) cells were treated for 24 h with different concentrations of 3 buttressed analogs of CIM-D-pPhOH containing 3-Cl, 3,5-Br₂ and 3-Cl-5-OCH₃ substituents, and whole cell lysates were analyzed by western blots for induction of p21, SERPINB5 and GADD45α.

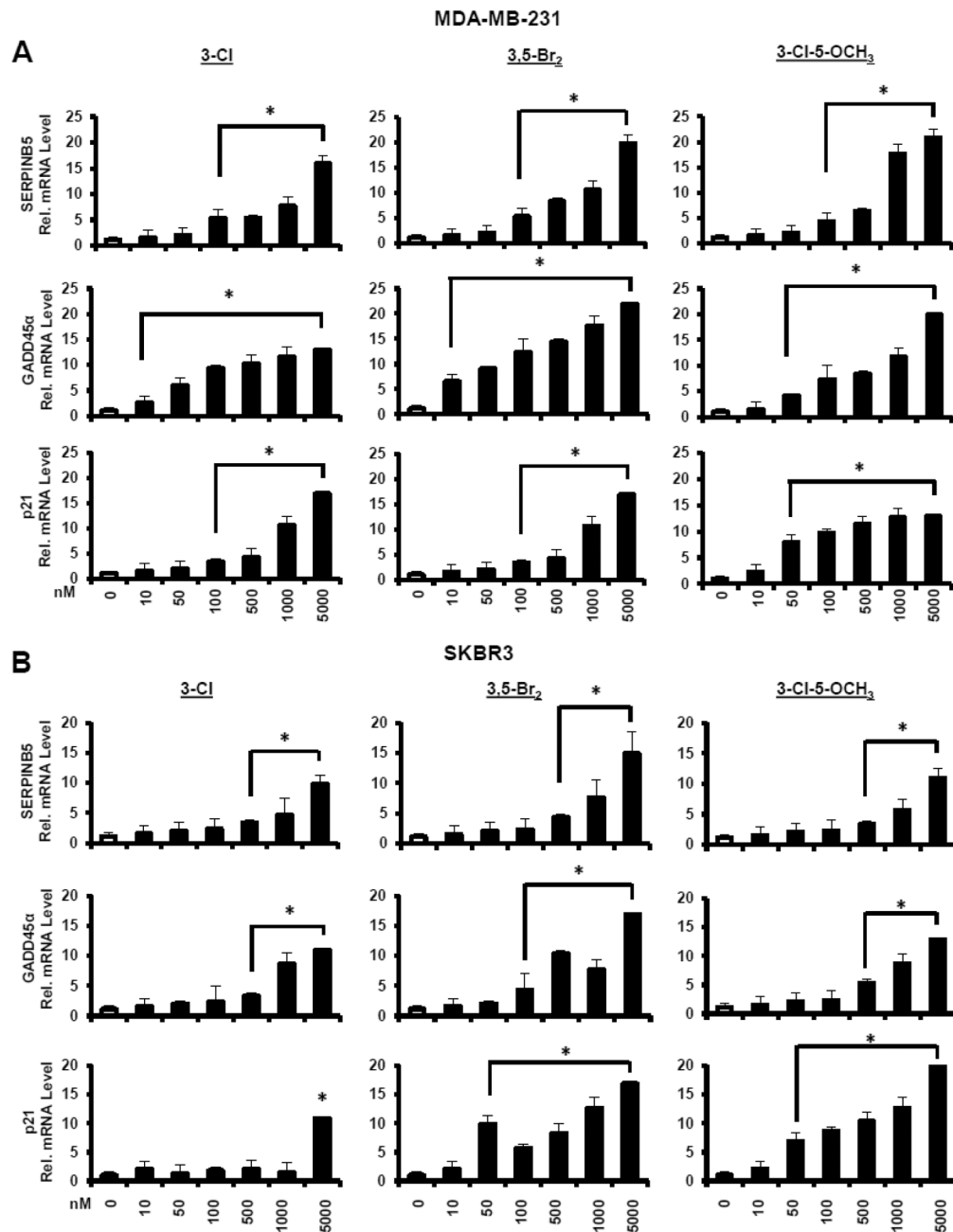


Figure 4.

Induction of gene expression by buttressed analogs in breast cancer cells. MDA-MB-231 (A) or SKBR3 (B) cells were treated for 12 h with different concentrations of 3-Cl, 3,5-Br₂ and 3-Cl-5-OCH₃ buttressed analogs of DIM-C-pPhOH, and induction of SERPINB5, GADD45α and p21 mRNA was determined by real time PCR. Results are expressed as means ± SE for 3 replicated determinations, and significant ($p < 0.05$) induction is indicated (*).

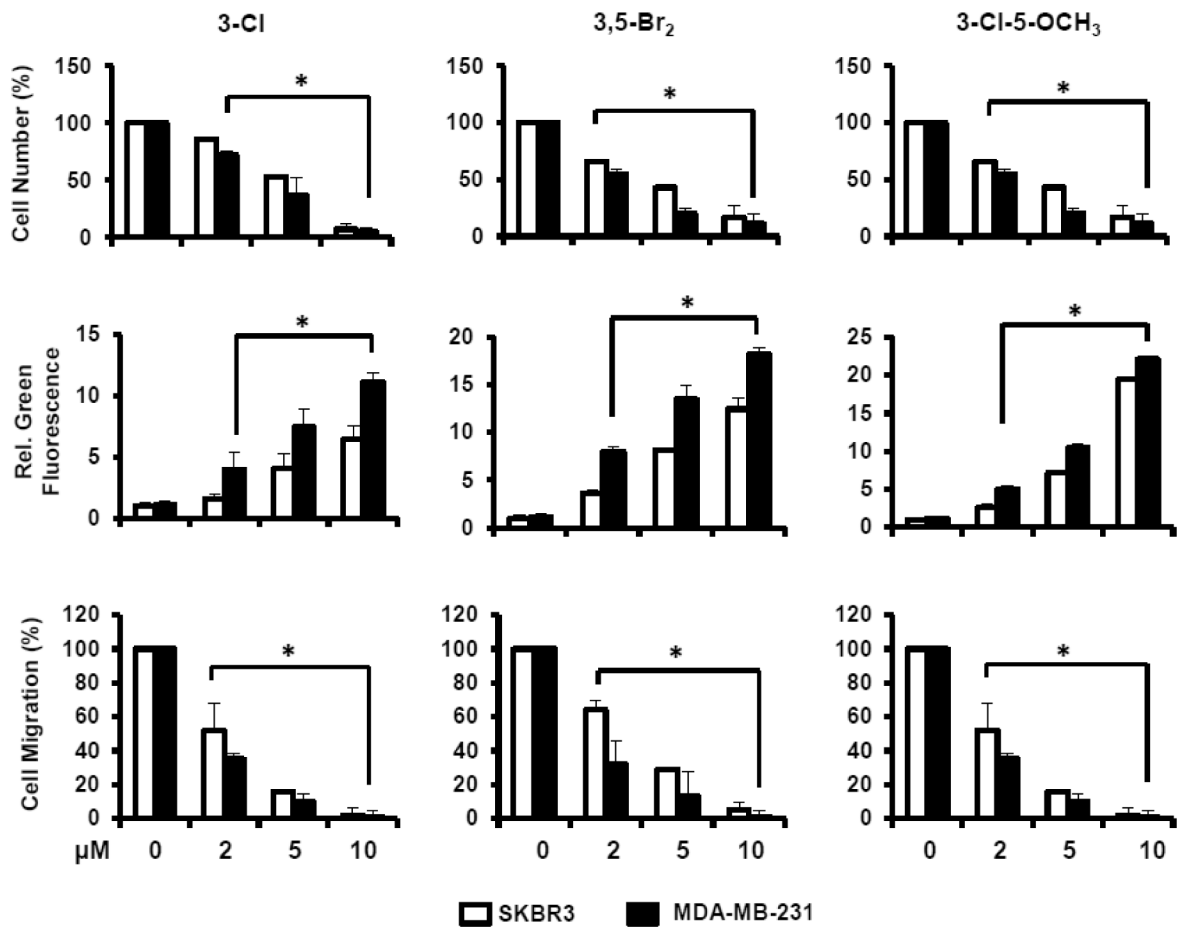


Figure 5.

Growth inhibition, induction of apoptosis and inhibition of MDA-MB-231 and SKBR3 cell migration by buttressed analogs. Cells were treated for 24 h with different concentrations of the 3-Cl, 3,5-Br₂ and 3-Cl-5-OCH₃ buttressed analogs of DIM-C-pPhOH, and effects on cell growth inhibition (A), induction of Annexin V staining (B), and inhibition of cell migration in a Boyden chamber assay (C) were determined as outlined in the Materials and Methods. Results are expressed as means \pm SE for at least 3 determinations for each treatment group and significant ($p < 0.05$) effects are indicated (*).

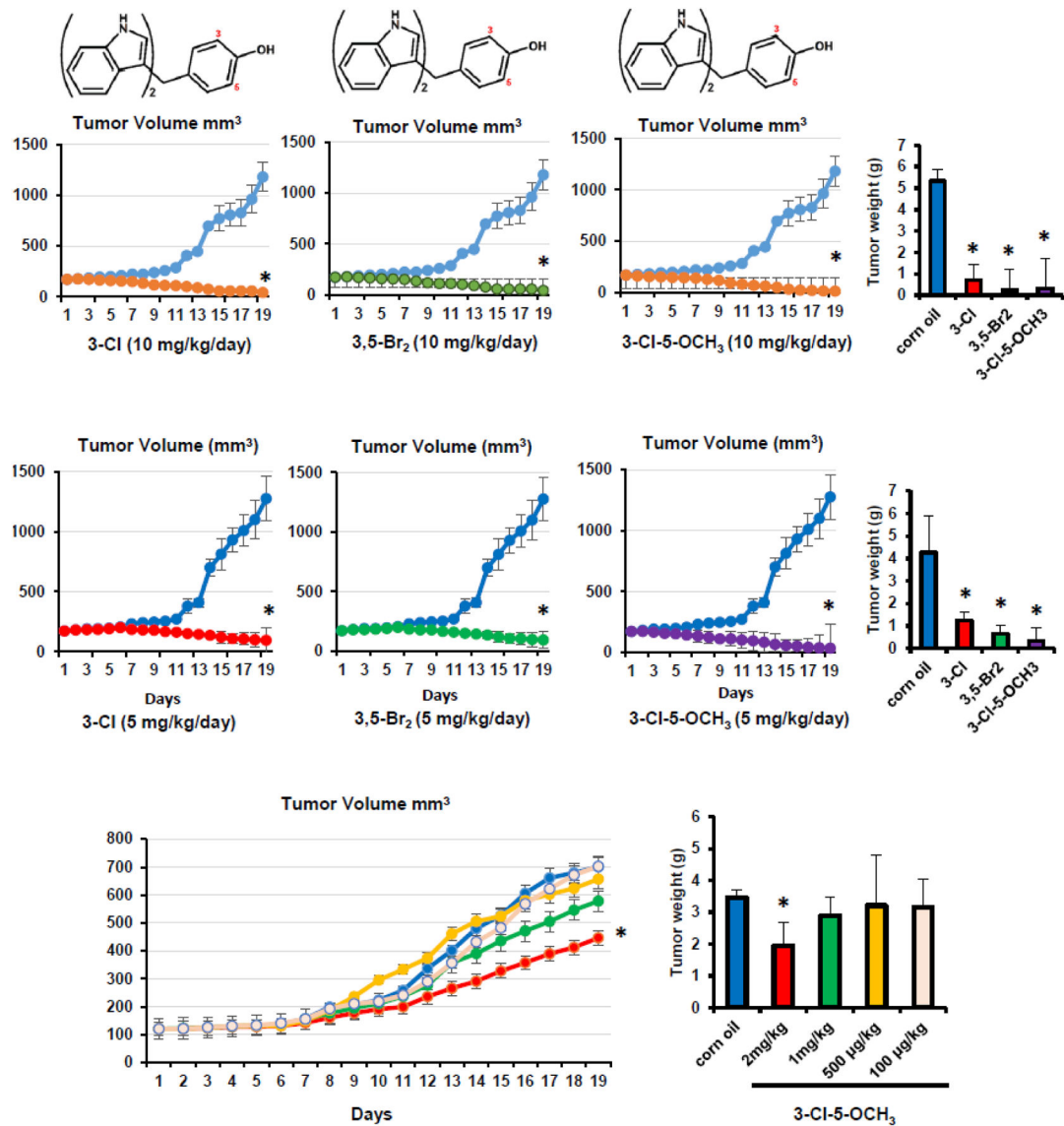


Figure 6. Buttressed analogs as inhibitors of breast tumor growth in athymic nude mice. The 3-Cl, 3,5-Br₂ and 3-CI-5-OCH₃ buttressed analogs of DIM-C-pPhOH were administered to athymic nude mice bearing MDA-MB-231 cells at doses of 10 mg/kg/d (A) and 5 mg/kg/d (B), and effects on tumor growth and weight were determined as outlined in the Materials and Methods. The effects of low doses (100 µg – 2 mg/kg/d) of 3-CI-5-OCH₃ on tumor growth (C) and (D) weight were determined as outlined in the Materials and Methods. At least 4 mice were used for each dose. Tumor volumes and weights are means ± SD, and significant (p < 0.05) effects are indicated (*).

Table 1.IC₅₀/EC₅₀ Values (μM)

	3-Cl	3,5-Br ₂	3-Cl-5-OCH ₃
Growth Inhibition	4.56 5.24	4.22 5.54	4.07 5.91
Annexin V Staining	5.34 6.46	4.79 5.82	3.39 4.76
Migration inhibition	2.67 4.02	2.56 3.88	2.11 3.56
SERPINB5 (induction)	1.25 3.01	0.89 2.54	0.54 1.28
GADD45α (induction)	1.78 322	0.77 2.47	0.63 1.36
p21 (induction)	3.89 4.97	0.45 1.21	0.19 1.09

MDA-MB-231

SKBR3