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The cellular and molecular effects of the androgen receptor agonist, CI-4AS-1, on breast cancer cells

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ABSTRACT

Purpose: The androgen receptor (AR) has attracted attention in the treatment of breast cancer. Due to the undesirable side effects of AR agonists, attempts have been undertaken to develop selective AR modulators. One of these compounds is CI-4AS-1. This study examined this compound more closely at the cellular and molecular levels.

Methods: Three different breast cancer cell lines were utilized, namely the luminal MCF-7 cells, the molecular apocrine MDA-MB-453 cells, and the triple negative, basal MDA-MB-231 cells.

Results: High and significant concordance between dihydrotestosterone (DHT) and CI-4AS-1 in regulation of gene expression in MDA-MB-453 cells was found. However, some differences were noted including the expression of AR, which was upregulated by DHT, but not CI-4AS-1. In addition, both DHT and CI-4AS-1 caused a similar morphological change and reorganization of the actin structure of MDA-MB-453 cells into a mesenchymal phenotype. Treatment of cells with DHT resulted in induction of proliferation of MCF-7 and MDA-MB-453 cells, but no effect was observed on the growth of MDA-MB-231 cells. On the other hand, increasing doses of CI-4AS-1 resulted in a dose-dependent inhibition on the growth of the three cell lines. This inhibition was a result of induction of apoptosis whereby CI-4AS-1 caused a block in entry of cells into the S-phase followed by DNA degradation.

Conclusions: These results indicate that although CI-4AS-1 has characteristics of classical AR agonist, it has dissimilar properties that may make it useful in treating breast cancer.

Introduction

Understanding the biology of breast cancer and the utilization of advanced molecular technology have enabled better differentiation and classification of this disease paving the road for targeted therapy and improving its treatment. For example, tamoxifen, a selective estrogen receptor modulator (SERM), has been effective in reducing recurrence and progression of hormone-responsive breast cancer while being beneficial for the treatment of osteoporosis. The use of aromatase inhibitors that block the conversion of androgens into estrogens has been reported to increase overall survival and progression among post-menopausal women. A breast cancer subtype that is marked by amplification of human epidermal growth factor receptor 2 (HER2), hence termed HER2-enriched breast cancer, is currently treated with trastuzumab, a humanized monoclonal antibody that inhibits the function of the receptor. Combinational therapy like trastuzumab and aromatase inhibitors has shown to be effective in therapy. Nevertheless, resistance toward these treatments is a hallmark of breast cancer therapy. Mutations in molecular component of other pathways such as PI3K/AKT/mTOR or cell cycle regulators compromise endocrine therapy. In addition, it is challenging to treat triple-negative breast cancer (TNBC), which lacks the expression of estrogen receptor (ER), progesterone receptor, and HER2, due to the absence of a target molecule. Making this worse is the fact that TNBC is actually a heterogeneous disease that is composed of multiple subtypes, each with a distinct molecular profile, behavior, and response to therapy.

In recent years, attention has been directed toward alternative therapeutic targets. One such target is
androgen receptor (AR). In fact, androgen therapy was a strategy of treating breast cancer decades ago till the emergence of targeted therapeutics such as SERMs and inhibitors of androgen aromatization into estrogen. There has recently been a revival of utilizing antiandrogen therapy due to a number of reasons. First of all, breast cancer is a heterogeneous disease with multiple subtypes, one of which is characterized by active AR signaling pathway and, hence, known a luminal AR and is represented by molecular apocrine breast cancer. In addition, AR is expressed in all subtypes of breast cancer, although the extent of expression varies according to the type. For example, up to 90% of luminal breast cancer is found to be AR-positive, relative to 32% of basal-like breast cancer. In fact, AR expression is associated with better prognosis of the disease, although it has been suggested that it can also act as an oncogene in ER-negative breast cancers. It has been shown that treatment of TNBC cells with AR antagonists reduces cell proliferation. On the other hand, contradictory results have been reported on the effect of AR agonists on breast cancer cells (for review, see). Nevertheless, clinical trials have shown promising results in treating breast cancer patients.

The undesirable effects of classical AR agonists such as testosterone or dihydrotestosterone (DHT) have geared scientists toward developing AR ligands that are tissue selective, hence termed, selective androgen receptor modulators (SARMs). These molecules act as mimics of AR agonists, but can regulate the activity of AR in tissues selectively reducing undesirable side effects (for review, see). Some success has been reported whereby SARMs possess anabolic effects on muscles without affecting other tissues. In addition, one such SARM was recently shown to inhibit the growth of TNBC cells. Cl-4AS-1 was first described by Tolman et al. as a potential SARM. It was later shown to have an agonistic effect similar to DHT both in vitro and in vivo and has recently been shown to bind AR and the complex translocates into the nucleus. Due to insufficient studies on this compound, we aimed to further analyze Cl-4AS-1 as an alternative AR agonist. In this study, the effect of Cl-4AS-1 on, primarily, a luminal AR breast cancer model system, MDA-MB-453 cells, was investigated. We reveal that Cl-4AS-1 possesses some functional resemblance to DHT, but it regulates some genes differently and induces cell death via blocking cell entry into the S phase of the cell cycle.

Methods

Cell lines

Three breast cancer cell lines, MDA-MB-453, MDA-MB-231, and MCF-7 cells were used in this study. The cells were maintained in a Leibovitz’s L-15, Dulbecco’s modified Eagle’s medium-high glucose, and RPMI media, respectively, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin-streptomycin. MDA-MB-453 cells were maintained in a humidified incubator at 37°C without CO₂. MDA-MB-231 and MCF-7 cells were maintained in a humidified incubator at 5% CO₂ and 37°C.

Compounds

DHT was obtained from Tokyo Chemical Industry (Tokyo, Japan), whereas Cl-4AS-1 was purchased from Tocris Inc. (Bristol, UK). Bicalutamide was obtained from Santa Cruz Biotechnology (Heidelberg, Germany). All compounds were prepared in dimethyl sulfoxide (DMSO), aliquoted, and stored at −20°C. The concentrations used herein were initially based on the previous study of Schmidt et al. In general, experiments were performed within 2 months of reconstitution of Cl-4AS-1 in DMSO.

Polymerase chain reaction (PCR) array

All reagents for the PCR arrays were obtained from Qiagen (Valencia, CA, USA). The PCR array was custom-made containing genes from different pathways including adhesion receptors, proteases and their inhibitors, signaling molecules, and apoptosis (Supplemental Table 1S). A gene ontology analysis was carried out using the web-based gene ontology database application DAVID (http://david.abcc.ncifcrf.gov). A level 5 search in the “biological process” category was carried out in order to provide the highest degree of specificity for target function.

MDA-MB-453 cells were plated onto 100-mm plates. Cells were treated with either 100 nM DHT or 1 µM Cl-4AS-1 for 24 and 72 h. Cells treated with DMSO were used as a negative control. Total
cellular RNA was extracted using RNeasy Mini Kit and treated with DNase I digestion according to manufacturer’s instructions. One microgram of RNA was converted to cDNA by RT² First Strand Kit according to manufacturer’s protocol. DNA amplification was performed using RT² qPCR Master Mix. PCR amplification was performed in a Bio-Rad’s IQ Real-Time System (Hercules, CA, USA). The reaction conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. At the completion of the reaction, $C_t$ values were determined, and $\Delta\Delta C_t$ and fold changes of expression were determined using the RT² Profiler PCR Array Data Analysis web portal based on the average expression of four housekeeping genes. Samples were analyzed in duplicates. Pearson correlation was used to compare changes in gene expression induced by DHT and Cl-4AS-1.

**Immunoblotting**

Cells were lysed in ice-cold lysis buffer (2% sodium dodecyl sulfate (SDS), 10 mM Tris pH 7.5, 10 mM NaF, 2 mM EDTA, 10 mM dithiothreitol). Proteins were resolved by electrophoresis on a 7.5% SDS-polyacrylamide gel and transferred onto 0.45-µm nitrocellulose membranes. Primary antibodies, rabbit anti-AR (Santa Cruz Biotechnology) and rabbit anti-GAPDH (Abcam, UK), were used followed by appropriate horseradish peroxidase (HRP)-conjugated-secondary antibodies. Detection was performed using Luminanta Crescendo Western HRP Substrate (Millipore, MA, USA). Images were captured using C-DiGit blot scanner (Licor Biosciences, Lincoln, NE, USA). Densitometric analysis was performed on the blots using Image Studio Digits v. 5.2 (Licor Biosciences).

**Cell proliferation (MTT) assay**

Cell proliferation assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT] assay) was performed according to the method of Mosmann with modifications.²⁷ Briefly, $7 \times 10^3$ viable cells were seeded in the wells of a 96-well tissue culture plate containing growth media supplemented with FBS. Cultures were treated at 24 h after seeding. Freshly prepared MTT) salt (5 mg/ml in phosphate-buffered saline (PBS)) was added to each well to give a final concentration of 0.5 µg/µl. The plates were incubated for 4 h and the formation of formazan crystals was checked using an inverted microscope. Equal volume of 1:1 (200 µl) of mix of DMSO and isopropanol was added to each well and incubated for 30–45 min. Cell viability was evaluated by measuring the absorbance at 570 nm using Sunrise microplate reader (Tecan, Switzerland).

**Morphology**

For morphology assays, MDA-MB-453 cells were seeded at $2 \times 10^6$ cells in 60-mm tissue culture dishes. Cells were grown overnight and then treated with either 1 µM Cl-4AS-1 or 100 nM DHT for 3 and 6 days in the presence or absence of 10 µM bicalutamide. Images were captured by DMIL LED inverted microscope (Leica Microsystems, Wetzlar, Germany).

**Immunofluorescence**

Cells (10,000 cells/well) were cultured on cover-slips coated with 10 µg/ml collagen type 1 solution (Sigma, USA). Following treatment, cells were fixed by incubation with 4% paraformaldehyde for 1 h and permeabilized with 0.5% Triton X-100/PBS for 10 min. Actin was stained by incubation with Alexa Fluor 488 phalloidin (Invitrogen, USA) for 1 h. The coverslips were mounted with ProLong Gold Antifade Mountant in the presence of DAPI (Invitrogen).

**Cell cycle analysis**

The cells were seeded in 100-mm plate at $1 \times 10^6$ cells/dish in growth media. The cells were allowed to attach overnight and then treated with either 1 or 4 µM Cl-4AS-1 for 3 or 6 days. The collected cells were then washed in PBS, fixed in 75% ethanol, then stained with 50 µg/mL propidium iodide containing 10 µg/mL RNase A. The cells were analyzed in a Miltenyi MACS Quant flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany). This instrument analyzes specified volume rather than recorded events.
Results

Selective regulation of gene expression by DHT and CI-4AS-1

A comparison of the regulation of gene expression by both CI-4AS-1 and DHT was first carried out. For this purpose, MDA-MB-453 cells were treated with CI-4AS-1 (1 µM) or DHT (100 nM) for 24 and 72 h. The concentrations were used based on the previous study of Schmidt et al.\textsuperscript{25} The expression of 85 genes related to adhesion receptors, proteases and their inhibitors, signaling molecules, and apoptosis was examined by PCR arrays. Changes of gene expression of more than twofold for the 24-h treatment by either AR agonist were considered, whereas differences of fivefold and higher for the 72-h treatment were considered since the latter treatment would be expected to have secondary or tertiary transcriptional effects. Upon assessing changes in the expression of genes, there was a high level of concordance of gene expression following treatment with either DHT or CI-4AS-1 with Pearson correlation coefficient ($R$) values of 0.90 and 0.93 after 24 and 72 h of treatment, respectively. This concordance was highly significant with a $p$-value of $<0.001$ for both correlations (Figure 1A and B). Such concordance is an indication of a similar effect of both DHT and CI-4AS-1 on gene expression. As shown in Table 1, 16 genes had an altered expression in cells treated with either DHT or CI-4AS-1. The fold change of expression was even comparable between the two treatments. For example, strong induction of gene expression was observed for metalloprotease 13 ($\text{MMP13}$) and integrin $\alpha_1$ ($\text{ITGA1}$) following 3 days of treatment. Interestingly, tenasin C ($\text{TNC}$) was the only gene that was consistently regulated by both treatments at both time points, where it was downregulated by five- to tenfold (Supplemental Table 1S, Table 2S).

Nonetheless, treatment-specific alterations of gene expression were also noted whereby 14 genes had an altered expression by one treatment, but not the other. For example, integrin $\beta_6$ ($\text{ITGB6}$) and $\text{MMP12}$ were both highly upregulated in cells treated by CI-4AS-1 only for 72 h. Interestingly, the effect of CI-4AS-1 and DHT on the expression of $\text{MMP10}$ was the opposite whereby the former compound downregulated the expression of this gene after 72 h of treatment, whereas DHT induced its expression.

DHT, but not CI-4AS-1, changes the expression of AR

We aimed then to investigate if CI-4AS-1 specifically changes the expression of AR protein. MDA-MB-453 cells were treated with either DHT (10 or 100 nM) or CI-4AS-1 (100 nM or 1 µM) for 24 or 72 h in the presence or absence of the AR antagonist, bicalutamide. As shown in Figure 2 and based on densitometric measurement, neither treatment altered the level of AR protein after 24 h. However, there was a two- to threefold increase in the level of AR protein upon treating the cells with DHT for 72 h, but not by CI-4AS-1. Bicalutamide negated the DHT-induced increase in AR protein levels.

Both DHT and CI-4AS-1 alter cell morphology

Interestingly, there was a dramatic change in the morphology of MDA-MB-453 cells with long-term treatment with either CI-4AS-1 or DHT. Normally,
Table 1. Alterations of gene expression following treatment of MDA-MB-453 cells with either Cl-4AS-1 (1 µM) or DHT (100 nM) for either 24 or 72 h.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cl-4AS-1 DHT</th>
<th>Cl-4AS-1 DHT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>72 h</td>
</tr>
<tr>
<td>ITGA1</td>
<td>1.4</td>
<td>1.3</td>
</tr>
<tr>
<td>ITGA5</td>
<td>-2.1</td>
<td>-2.4</td>
</tr>
<tr>
<td>ADAM9</td>
<td>-1.1</td>
<td>1.2</td>
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<td>ADAM17</td>
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<tr>
<td>MMP13</td>
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<td>-1.7</td>
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<tr>
<td>ADAMT515</td>
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<td>EPHA4</td>
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<tr>
<td>ERBB2</td>
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</tr>
<tr>
<td>CADM2</td>
<td>-1.4</td>
<td>-1.2</td>
</tr>
<tr>
<td>CTNNB1</td>
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<td>-1.1</td>
</tr>
<tr>
<td>CXCR7</td>
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<td>-4.6</td>
</tr>
<tr>
<td>FOXA1</td>
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<td>-2.1</td>
</tr>
<tr>
<td>ZEB1</td>
<td>LE</td>
<td>LE</td>
</tr>
<tr>
<td>FOXO1</td>
<td>-3.0</td>
<td>-2.0</td>
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<tr>
<td>TNC</td>
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<td>-9.6</td>
</tr>
<tr>
<td>CASP9</td>
<td>-1.2</td>
<td>-1.3</td>
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Genes either concordantly upregulated or downregulated by both treatments at either duration

<table>
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<tr>
<th>Gene</th>
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<th>Cl-4AS-1 DHT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>72 h</td>
</tr>
<tr>
<td>ITGB5</td>
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<tr>
<td>ITGB6</td>
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<tr>
<td>ADAM2</td>
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<tr>
<td>ADAM15</td>
<td>2.1</td>
<td>1.7</td>
</tr>
<tr>
<td>MMP12</td>
<td>1.4</td>
<td>-1.1</td>
</tr>
<tr>
<td>MMP16</td>
<td>-1.9</td>
<td>-2.6</td>
</tr>
<tr>
<td>ADAMT518</td>
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</tr>
<tr>
<td>CTSB</td>
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<td>1.2</td>
</tr>
<tr>
<td>CDH1</td>
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<td>-1.7</td>
</tr>
<tr>
<td>CD44</td>
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<td>LE</td>
</tr>
<tr>
<td>IGFR1</td>
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<tr>
<td>CCL5</td>
<td>-1.4</td>
<td>-2.3</td>
</tr>
<tr>
<td>CASP10</td>
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</tr>
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</table>

Genes either upregulated or downregulated by one treatment only at either duration

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Cl-4AS-1 DHT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>72 h</td>
</tr>
<tr>
<td>MPP10</td>
<td>1.3</td>
<td>-1.0</td>
</tr>
</tbody>
</table>

*Changes of differential expression of more than two folds for either treatment at 24 h and more than five folds for either treatment at 72 h were considered and are in bold.
LE = low expression (appearing at cycle >32) in both control and treated samples.

The cells appeared rounded forming colonies of cell clusters with cells growing on top of each other. Upon treating the cells with either 1 µM Cl-4AS-1 or 100 nM DHT, the cell shape was altered dramatically where, after 3 days of treatment, cell clustering was reduced and the cells had a spindle shape (Figure 3A, C, and E). Treatment of cells with lower concentrations of Cl-4AS-1 (100 nM) or DHT (10 nM) also resulted in a similar change (data not shown). Prolonged treatment (6 days) caused a more pronounced change in cell morphology (Figure 3B, D, and F). At both periods, the change in cell morphology by DHT (Figure 3I and J) or Cl-4AS-1 (Figure 3K and L) was inhibited by co-treating cells with 10 µM of bicalutamide (Figure 3G and H). A combinational treatment of cells with both Cl-4AS-1 and bicalutamide reduced the change in cell morphology at day 3, but not at day 6. During the prolonged treatment, though, there was an increase in cell clustering indicating an inhibitory effect of bicalutamide on the function of Cl-4AS-1.

The alteration of cell morphology may have reflected actin reorganization. Indeed, cells treated with either 1 µM of Cl-4AS-1 or 100 ng of DHT had an increase in filamentous actin compared to control cells, which had actin concentrated at the cell cortex and regions of cell–cell contact (Figure 4). Additionally, cells treated with DHT showed a metastable cell phenotype. On the other hand, MDA-MB-453 cells exposed to Cl-4AS-1 had intense filamentous actin staining and formation of lamellipodia. Cl-4AS-1-treated cells were also spindle in shape and had a front–back polarity, characteristics of the mesenchymal phenotype.

**Cl-4AS-1, but not DHT, suppresses cell growth**

The effect of both DHT and Cl-4AS-1 on cell proliferation was investigated on MCF-7, MDA-MB-453, and MDA-MB-231 cells. The three cell lines expressed AR at variable levels with MDA-MB-453 expressing the highest levels and MDA-MB-231 cells expressing the least (data not shown). The cells were treated with increasing concentrations of both DHT (1, 10, 100, and 1000 nM) and Cl-4AS-1 (0.1, 1, 5, and 10 µM) and cell proliferation was measured after 3, 5, and 7 days.
Figure 3. AR-dependent morphological alteration of MDA-MB-453 cell line by Cl-4AS-1 and DHT. MDA-MB-453 cells were either untreated (A and B) or treated with 100 nM DHT (C and D), 1 µM Cl-4AS-1 (E and F), 10 µM bicalutamide only (G and H), 100 nM DHT and bicalutamide (10 µM; I and J), and Cl-4AS-1 (1 µM) and bicalutamide (10 µM; K and L) for 3 (A, C, E, G, I, and K) or 6 (B, D, F, H, J, and L) days. Magnification was set at 20×. Experiment was done in triplicate.
Disparate effect of DHT was observed on the three cell lines (Figure 5A, C, and E). Whereas it stimulated growth of MCF-7 cells at increasing doses (Figure 5A), it induced the proliferation of MDA-MB-453 cells at all concentrations at a similar extent (Figure 5C), and did not have any effect on the growth of MDA-MB-231 cells (Figure 5E). The effect of DHT on the growth of cells started at day 3, but it became more appreciable at longer treatments. On the other hand, increasing concentrations of Cl-4AS-1 suppressed the proliferation of the three cell lines similarly with complete inhibition at a concentration of 10 µM (Figure 5B, D, and F). The SARM also had a similar inhibitory effect on two other breast cancer cell lines, ZR75-1 and T47D (data not shown). The effect of Cl-4AS-1 on cell growth was initially apparent on day 3. It was also noted that as preparations of the compound became older, its effect became less. However, the molecular and cellular effects sustained.

Cl-4AS-1 induces apoptosis via blocking entry into S phase

We decided to elucidate the mechanism of cell death induced by Cl-4AS-1. Cell cycle analysis was carried out on MDA-MB-453 cells treated with 1 and 4 µM of Cl-4AS-1 for 3 and 6 days. As illustrated in Figure 6, a block in the early S phase was observed in cells treated with 4 µM Cl-4AS-1 for 3 days relative to both the control cells and those treated with 1 µM (Figure 6A–C). A large fraction of the cells treated with 4 µM Cl-4AS-1 was detected in the sub-G phase indicating DNA degradation and cell death (Figure 6C). After 6 days of treatment, cells treated with 1 µM Cl-4AS-1 had a similar block in the S phase and an increase in apoptotic cells (Figure 6E).

Discussion

The AR agonist, Cl-4AS-1, was previously shown to act in a similar manner as DHT in terms of its effects on gene expression, bone formation, and uterus weight. The latter study has also illustrated that DHT and Cl-4AS-1-induced alterations in gene expression in MDA-MB-453 cells correlated by approximately 90%. We similarly found a strong correlation in changes in gene expression between Cl-4AS-1 and DHT in MDA-MB-453 cells. However, both this study and that of Schmidt et al. have found a degree of differential effect in modulating gene expression by both AR agonists necessitating caution when interpreting results using Cl-4AS-1. The change in cell morphology upon treating MDA-MB-453 cells with either AR agonist also illustrates that they function via a common mechanism. Such an effect is observed despite the fact that the AR gene possesses a mutation that reduces its transcriptional activity. The transformation in cell morphology was reversed by co-treating the cells with the AR inhibitor, bicalutamide. However, pronounced effect of Cl-4AS-1 on the actin cytoskeleton and the lesser inhibitory effect of the antagonist on this SARM could be due to the use of a smaller ratio of compound:inhibitor in case of Cl-4AS-1 (1:10) than that of DHT (1:100). Unfortunately, using a larger ratio of Cl-4AS-1:bicalutamide would require the use higher concentration of the antagonist, which was toxic to the cells.

Whereas DHT has a stimulatory effect on proliferation of MCF-7 and MDA-MB-453, Cl-4AS-1 inhibited proliferation of all breast cancer cell lines, including two other ones (T47D and ZR75-1), regardless of the subtype. It is not clear whether Cl-4AS-1 acts via the same pathway that suppresses cell
growth in the different breast cancer cell types or not. SARMs, similar to SERMs, have a tendency to selectively bind coregulators.\textsuperscript{29} Such selectivity is governed by the conformational change induced by the natural ligand versus the tissue-selective modulator, the coactivator:corepressor ratio in cells, and the existence of external stimuli. In a previous study, we have shown that Cl-4AS-1 differentially alters expression of microRNA molecules in breast cancer cell lines including two of the same subtype.\textsuperscript{30} Unfortunately, no other molecular study has been conducted on this compound.

Cl-4AS-1 loses its apoptotic efficacy with longer storage, although its behavior as a DHT analog is preserved. We observed that at a concentration of 10 \( \mu \text{M} \), the compound was completely lethal when freshly prepared. This concentration was used by Schmidt et al. when testing its transactivation of AR.\textsuperscript{25} Apparently, Cl-4AS-1 lost its lethal effect in the aforementioned study at the time of performing the experiment. It is not known how Cl-4AS-1 loses its killing properties. It can be hypothesized that the modifying group at carbon 17\textsuperscript{24} is cleaved making it structurally similar to DHT. This group

Figure 5. Suppression of cell growth by Cl-4AS-1 but not by DHT in breast cancer cell lines. MCF-7 (A and B), MDA-MB-453 (C and D), and MDA-MB-231 (E and F) cells were treated with increasing concentrations of either DHT (A, C, and E) or Cl-4AS-1 (B, D, and F). As indicated in the legends, the concentrations used for DHT were 0, 1, 10, 100, and 1000 nM, whereas the concentrations used for Cl-4AS-1 were 0, 0.1, 1, 5, and 10 \( \mu \text{M} \). Cell proliferation was measured after 3, 5, and 7 days of treatment. The experiment was repeated at least three times in quadruplicates.
is linked to the D ring of Cl-4AS-1 via an amide bond, which was shown in other SARMs to be hydrolyzed.\textsuperscript{31} Further investigation is warranted to analyze any structural modification of Cl-4AS-1.

The mechanism of action of Cl-4AS-1 in inhibiting cell proliferation appeared to be blocking the cell cycle at the early S phase inducing DNA degradation and apoptosis with continuing blocking. It is not clear, however, what the molecular mechanism of this SARM exactly is, but the arrest could be due to a stalemate at any of the processes of DNA replication such as inhibition of histone synthesis, enzyme activities like DNA polymerases and topoisomerases, and uncoiling of chromatin. Such phenomenon does not seem to be restricted to Cl-4AS-1. Yu et al. have recently reported that the SARM, RAD140, down-regulated the expression of genes associated with cell division, DNA replication, and cell cycle progression.\textsuperscript{32} In addition, a SARM, MK04541, has previously been reported to induce cell death specifically in AR-positive prostate cancer cells via induction of caspase 3.\textsuperscript{33} Another SARM, termed GTx-024, was found to inhibit the growth of AR-expressing MDA-MB-231 cells in vitro and in vivo.\textsuperscript{25} The latter SARM also reduced the release of two invasion-promoting factors, interleukin 6 and matrix metalloproteases 13 (MMP13), in correlation with reduced cell invasion and metastasis. Interestingly, we have recently reported that the expression of MMP13 is upregulated in MDA-MB-453 cells treated with DHT.\textsuperscript{30} The SERM, raloxifene, induces apoptosis in prostate cell lines following modulation of both transcriptional regulation and non-genomic signals via activation of ER\(\beta\) and antagonism of ER\(\alpha\).\textsuperscript{34}

The inhibitory effect of treating breast cancer cells with anti-AR has resulted in re-gaining interest in targeting AR in breast cancer. This is particularly true considering that some types of breast cancer are stimulated to grow in the presence of AR agonists\textsuperscript{35}, and that treatment of breast cancer cells with AR antagonists results in decreased cell growth.\textsuperscript{16,36} Future use of AR antagonists in treating breast cancer is potentially promising for two types of breast cancers. The first class is ER-positive cancers that develop resistance to neoadjuvant endocrine therapy. Although the high expression of AR is associated with having better response toward ER-based therapies\textsuperscript{37}, the overexpression of AR has been
found to increase resistance of cells toward tamoxifen.\textsuperscript{38} In addition, a recent study has shown that taking into consideration both AR and ER expressions in breast cancer cells can be a more accurate method for determining response to tamoxifen treatment.\textsuperscript{39} In this study, overexpression of AR relative to ER is associated with failure to respond to tamoxifen and poor survival in ER-positive breast cancers. The same study also illustrates that treatment of this tumor type with AR antagonists results in decreased cell proliferation. Specifically, one antagonist, enzalutamide, inhibits both ER- and AR-stimulated cell growths in vivo and in vitro. In our case, DHT induces the growth of ER-positive MCF-7 cells similar to what has been shown before\textsuperscript{40–44} indicating that, even in cells that have not developed resistance toward endocrine therapy, inhibition of AR signaling can be beneficial.

The second type of breast cancers that can be targeted by AR antagonists is TNBC, particularly, those known as molecular apocrine tumors that are characterized by being ER-negative, AR-positive cells. In this breast cancer subtype and using MDA-MB-453 cell line as a model system, AR tends to have the same oncogenic effect of ER via promoting cell proliferation\textsuperscript{40} and binding to chromatin regions that are characteristic of ER-binding sites.\textsuperscript{14} In this study, we have observed an increase in the proliferation of the same cell line upon treating them with DHT. Blocking AR signaling via treatment of these cells with bicalutamide or reducing the expression of AR results in slower growth and the formation colonies.\textsuperscript{14} Interestingly, targeting AR with inhibitors such as bicalutamide in TNBC has shown inhibitory effect on cell growth and tumorigenecity, even though they do not have an expression profile of active AR signaling.\textsuperscript{16,45} A number of clinical trials based on blocking the action of AR or androgen synthesis in AR-positive breast cancer are currently underway.\textsuperscript{46}

\section*{Conclusion}

Collectively, CI-4AS-1 can be used as an alternative agonist to classical AR agonists such as DHT. However, it is recommended to be used at lower concentrations to prevent induction of apoptosis. Our results also indicate a novel mechanism by which CI-4AS-1 induces death of breast cancer cells regardless of their subtypes. This effect could be induced via interaction with different co-regulators than DHT or at different affinities resulting in modulating divergent signaling pathways and gene expression profiling. Hence, the molecular mechanism of action of this SARM needs to be elucidated. However, the stability of the compound needs to be improved in order to generate reproducible results and to be considered for in vivo studies. In addition, the fact that DHT causes a stimulation of some AR-expressing breast cancer cells signifies the utilization of AR antagonists in the treatment of this cancer type.

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\section*{Declaration of interest}

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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