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Selective activity of deguelin identifies therapeutic targets for androgen receptor-positive breast cancer

Andrew J. Robles¹, Shengxin Cai^{3,4}, Robert H. Cichewicz^{3,4}, and Susan L. Mooberry^{1,2}

¹Department of Pharmacology, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas 78229-3900, United States

²Cancer Therapy & Research Center, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas 78229-3900, United States

³Natural Product Discovery Group, Institute for Natural Products Applications and Research Technologies, University of Oklahoma, 101 Stephenson Parkway, Norman, Oklahoma 73019, United States

⁴Department of Chemistry & Biochemistry, Stephenson Life Science Research Center, University of Oklahoma, 101 Stephenson Parkway, Norman, Oklahoma 73019, United States

Abstract

Triple negative breast cancers (TNBC) are aggressive malignancies with no effective targeted therapies. Recent gene expression profiling of these heterogeneous cancers and the classification of cell line models now allows for the identification of compounds with selective activities against molecular subtypes of TNBC. The natural product deguelin was found to have selective activity against MDA-MB-453 and SUM-185PE cell lines, which both model the luminal androgen receptor (LAR) subtype of TNBC. Deguelin potently inhibited proliferation of these cells with GI₅₀ values of 30 nM and 61 nM, in MDA-MB-453 and SUM-185PE cells, respectively. Deguelin had exceptionally high selectivity, 197 to 566-fold, for these cell lines compared to cell lines representing other TNBC subtypes. Deguelin's mechanisms of action were investigated to determine how it produced these potent and selective effects. Our results show that deguelin has dual activities, inhibiting PI3K/Akt/mTOR signaling, and decreasing androgen receptor (AR) levels and nuclear localization. Based on these data, we hypothesized that the combination of the mTOR inhibitor rapamycin and the antiandrogen enzalutamide would have efficacy in LAR models. Rapamycin and enzalutamide showed additive effects in MDA-MB-453 cells, and both drugs had potent antitumor efficacy in a LAR xenograft model. These results suggest that the combination of antiandrogens and mTOR inhibitors might be an effective strategy for the treatment of androgen receptor-expressing TNBC.

Robert H. Cichewicz, Tel: +1 (405) 325-6969 Fax: +1 (405) 325-6111. Rchichewicz@ou.edu, Susan L. Mooberry, Tel: +1 (210) 567-4788. Fax: +1 (210) 567-4300. Mooberry@uthscsa.edu.

Conflict of Interest

The authors declare that they have no conflict of interest.

Keywords

Triple negative breast cancer; deguelin; luminal androgen receptor; natural products; mTOR; enzalutamide

Introduction

Triple-negative breast cancers (TNBC) lack detectable expression of estrogen and progesterone receptors (ER/PR) and do not overexpress or have gene amplification of human epidermal growth factor receptor 2 (HER2/ERBB2) [1–4]. These heterogeneous cancers, which comprise approximately 10–15% of all breast cancers, are usually highly aggressive and patients have a poorer prognosis than patients with ER/PR positive disease [5–9]. While the epidemiology of TNBC is not understood, they are more common in African American women than Caucasians, and are typically diagnosed at an earlier age than other breast cancers [9–14]. While targeted therapies for ER/PR positive and HER2-amplified breast cancers have greatly improved patient survival, there are no molecularly targeted therapies available for TNBC and no therapies that provide long-term remission from metastatic TNBC [15–24]. The identification of molecular targets for effective treatment of TNBC requires a better understanding of the subtypes of these heterogeneous diseases and their molecular characteristics [25, 26]. Gene expression profiling and clustering analysis of 587 TNBC patient tumor samples by Lehmann and Bauer identified 6 molecular subtypes of TNBC, each with distinct molecular alterations and phenotypic characteristics [27]. Their classifications include two basal-like (BL1 and BL2), mesenchymal-like (ML), mesenchymal stem-like (MSL), luminal androgen receptor (LAR) and immunomodulatory (IM) subtypes. The number of subtypes is consistent with previous hierarchical clustering analysis by Kreike and colleagues showing five TNBC subtypes [28]. Lehmann and Bauer also identified cell line models for each TNBC subtype [27], providing the first *in vitro* models to screen for subtype-specific drug leads for TNBC.

Despite the lack of therapies for treating TNBC subtypes, recent studies have demonstrated that LAR TNBC cells are sensitive to a particular subset of chemotherapeutic agents. Lehmann and Bauer *et al* were first to show that cell lines and xenografts representative of this subtype are sensitive to both androgen receptor (AR) antagonists and heat shock protein 90 (Hsp90) inhibitors [27, 29], suggesting that targeting these proteins might be an effective treatment strategy. In addition to AR expression, analysis of patient data identified a high frequency of activating mutations in phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) in LAR TNBCs, and preclinical studies demonstrated that LAR cell lines are sensitive to phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) inhibitors *in vitro* [29]. These data suggest that inhibition of AR activity, PI3K signaling or potentially both might be effective for treating LAR TNBCs. However, evidence for the benefits of PI3K inhibitors and AR antagonists compared to other anticancer agents, particularly for the LAR subtype, is lacking.

Nature has provided a majority of the drugs used by humans throughout their history, and natural products continue to be a major source of new drug leads [30, 31]. Many of the most

effective drugs used today for cancer treatment are themselves natural products, or derived from a natural product pharmacophore [32, 33]. The microtubule-targeting agents paclitaxel, docetaxel, and the vinca alkaloids are still semi-synthetically derived from biological source materials. Natural products have distinct chemical properties compared to synthetic molecules, typically possessing more chiral centers and oxygen atoms than purely synthetic compounds [34]. This is biologically important because nearly all biomolecules utilized as drug targets are chiral. The co-evolution of plants and humans has resulted in plants producing secondary metabolites that are primed to interact with biological targets. For these reasons and others, we conducted screens of natural product libraries to identify extracts with selective activity against cell lines representing the TNBC molecular subtypes. We hypothesized that based on the different molecular characteristics of each TNBC subtype, compounds with selective antiproliferative or cytotoxic activity against specific TNBC subtypes could be identified. In this study, we report the isolation and identification of deguelin as a selective inhibitor of the LAR subtype of TNBC and demonstrate how mechanistic insights gleaned from mode of action studies of this natural product identified a combination of potential molecular targets for the LAR subtype of TNBC.

Methods

General Reagents

Authentic (–)-deguelin, rapamycin for *in vitro* studies and enzalutamide/MDV3100 were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Rapamycin for animal studies was purchased from LC Laboratories (Woburn, MA, USA). R1881 was purchased from Perkin Elmer (Waltham, MA, USA). Sulforhodamine B salt, paclitaxel, 17-AAG, crystal violet, Trizma, Dulbecco's phosphate-buffered saline (DPBS), HEPES, hydrocortisone, insulin, phenylmethanesulfonyl fluoride (PMSF), carboxymethyl cellulose, Tween-80, polyethylene glycol 400 and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Compounds for cell treatments were dissolved in DMSO and stored at –20°C. Compounds for *in vivo* studies were stored as stock solutions in DMSO (enzalutamide) or ethanol (rapamycin) at –20°C and diluted immediately before use.

Cell Culture

MDA-MB-453, MDA-MB-231, MDA-MB-468, HCC1937, HCC70, SK-BR-3 and LnCAP cells were purchased from the American Type Culture Collection (Manassas, VA, USA). BT-549 cells were obtained from Lombardi Comprehensive Cancer Center of Georgetown University (Washington, DC, USA) and the identity was validated by Promega (Fitchburg, WI, USA). SUM-185PE cells were purchased from Asterand Bioscience (Detroit, MI, USA). MDA-MB-453, MDA-MB-231 and SK-BR-3 cells were cultured in Improved Minimum Essential Medium (IMEM; Gibco, Waltham, MA, USA) with 10% fetal bovine serum (FBS; GE Healthcare, Little Chalfont, United Kingdom) and 25 µg/mL gentamicin (Gibco). MDA-MB-468, HCC1937, HCC70, BT-549 and LnCAP cells were cultured in RPMI-1640 medium (Sigma-Aldrich) with 10 % FBS and 50 µg/mL gentamicin. SUM-185PE cells were cultured in Ham's F-12 Nutrient Mix (Gibco) with 5% heat-inactivated FBS, 10 mM HEPES, 1 µg/mL hydrocortisone and 5 µg/mL insulin. Cells were maintained in humidified incubators at 37°C with 5% CO₂. All cell lines were initially

expanded and frozen as stocks in liquid nitrogen. Cells used in all assays were passaged for no more than 3 months after revival from liquid nitrogen.

Sulforhodamine B Assay

The antiproliferative/cytotoxic effects of all compounds after 48–96 h treatments were evaluated using the sulforhodamine B (SRB) assay as previously described [35, 36]. Cell number at the time of treatment (T_0) was measured to assess cytotoxic activity. Concentration-response curves were plotted and the concentrations causing 50% inhibition of proliferation compared to vehicle control (GI_{50}), total growth inhibition (TGI) and 50% cell death compared to T_0 (LC_{50}), were interpolated from nonlinear regressions of the data (Prism 6; GraphPad Software, La Jolla, CA, USA).

Colony formation assays

MDA-MB-453 cells were seeded at 500 cells/60 mm³ tissue culture dish, allowed to adhere overnight, then treated with vehicle or predetermined concentrations of deguelin. After 8 h or 24 h treatments, cells were washed with DPBS and fresh growth medium added. After 14 days, cells were fixed and stained with crystal violet. Colonies were counted using GeneSnap software (PerkinElmer). Data were analyzed by one-way ANOVA with Tukey's post-hoc test using Prism 6.

Whole-cell lysis and immunoblotting

Cells were treated with vehicle/deguelin, harvested by scraping and lysed in Cell Extraction Buffer (Life Technologies, Waltham, MA, USA) containing protease inhibitor cocktail, PMSF and Na_3VO_4 (Sigma-Aldrich). The total protein in each sample was measured with a Pierce Coomassie Plus Assay Kit (Life Technologies) and equal amounts of protein were separated by SDS-PAGE on NuPAGE Bis-Tris gels (Life Technologies). Proteins were transferred to PVDF membranes (EMD Millipore, Billerica, MA, USA) and probed with antibodies for actin (Sigma-Aldrich), P-mTOR, mTOR, P-S6K, S6K, P-RPS6, RPS6, P-4E-BP1, 4E-BP1, P-S473-Akt, Akt or androgen receptor (Cell Signaling Technology, Danvers, MA, USA) diluted in RapidBlock (AMRESCO, Solon, OH, USA). Chemiluminescence was visualized with SuperSignal West Pico Chemiluminescent Substrate (Life Technologies) using a Geliance imaging system (PerkinElmer) or Odyssey FC (LI-COR Biosciences, Lincoln, NE, USA).

Nuclear/cytoplasmic extraction

MDA-MB-453 cells were cultured in IMEM containing phenol red and 10% FBS or phenol red-free IMEM containing 10% dextran/charcoal-treated FBS (GE Healthcare) for 3 days to remove androgens. Cells were treated with compounds/vehicle for the indicated time periods, washed with DPBS and nuclear/cytoplasmic extracts were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Life Technologies) according to the manufacturer's instructions. The protein content of each sample was quantified and equal amounts of protein were separated by SDS-PAGE and evaluated by immunoblotting with antibodies against AR (Cell Signaling), GAPDH (Cell Signaling) or lamin B1 (Abcam).

Xenograft studies in nude mice

Male athymic nude mice (Harlan Laboratories, Indianapolis, IN, USA) were injected *s.c.* with 2×10^6 MDA-MB-453 cells, suspended in 100 μ L DPBS and 100 μ L Matrigel® (BD Biosciences, San Jose, CA, USA), bilaterally into each flank. After tumors reached a minimum volume of 150 mm³, mice were assigned to four groups (n = 10 tumors/group). Enzalutamide (5 mg/kg) was administered daily by oral gavage in a vehicle of 1% carboxymethyl cellulose, 0.1% Tween-80 and 5% DMSO in water (300 μ L total). Rapamycin (6.25 or 3.5 mg/kg) was administered every other day by *i.p.* injection in a vehicle of 4% ethanol, 5% polyethylene glycol 400, and 5% Tween-80 in DPBS (200 μ L total injection). Mice were weighed and examined daily for signs of toxicity. Tumors were measured twice weekly using calipers and tumor volume (mm³) was calculated as length (mm) $\times$ width (mm) $\times$ height (mm). At trial conclusion, tumors were excised, flash frozen in liquid nitrogen and weighed with an analytical balance. During the second xenograft trial, one mouse in the enzalutamide/vehicle group died 3 days after treatment began, due to a bacterial infection. Data are not included for this mouse after day 22. All mice were housed in an AAALAC-approved facility at The University of Texas Health Science Center at San Antonio and procedures were IACUC approved.

Tumor lysis and immunoblotting

Tumor tissue extracts from 3 mice in each treatment group were prepared. Entire tumors were flash frozen in liquid nitrogen, homogenized in Cell Extraction Buffer containing protease and phosphatase inhibitors and extracted for 2 h at 4 °C with gentle rotation. Lysates were cleared by centrifugation and total protein content was measured. SDS-PAGE and immunoblotting for P-RPS6, RPS6, P-S6K and S6K were performed as described for whole-cell lysis.

Statistical Analyses

Statistical analyses were conducted with GraphPad Prism 6. All *p* values reported are adjusted for multiple comparisons.

Results

Isolation of the plant-derived compound deguelin

A crude extract of *Amorpha fruticosa* exhibited selective antiproliferative activity against MDA-MB-453 cells, a model of the LAR subtype, relative to cell lines representing 4 other TNBC subtypes. Bioassay-guided fractionation on silica gel VLC and HP20ss columns, followed by C₁₈ preparative HPLC and C₁₈ semi-preparative HPLC identified deguelin as the active compound in this extract. The chemical structure (Fig. 1a) was determined based on comparisons of the ¹H and ¹³C NMR, specific rotation value, and MS data to published literature [37]. Deguelin is structurally related to the broad-spectrum toxin rotenone, used in a variety of insecticides and other poisons. [38–40]. The cytotoxic activity of deguelin against a variety of cancer cell lines, with potency in the low to mid-micromolar range, has been described [41, 42], but its high degree of selectivity for this cell line and low nanomolar potency has not been previously reported. We obtained deguelin from a commercial source,

confirmed that its structure matched the natural product, and confirmed its selective activity for MDA-MB-453 cells. Thereafter, the commercially-obtained deguelin was used for biological experiments.

Antiproliferative and cytotoxic activity of deguelin against TNBC molecular subtypes *in vitro*

The activity of deguelin was evaluated in a panel of TNBC cell lines representing 5 molecular subtypes of TNBC: MDA-MB-453, BT-549, MDA-MB-231, HCC1937, and HCC70 (Fig. 1b). These cells have been shown to model the molecular characteristics of the LAR, M, MSL, BL1 and BL2 subtypes defined by Lehmann and Bauer *et al*, respectively [27]. Deguelin had highly selective antiproliferative activity in MDA-MB-453 cells, representing the LAR subtype, relative to 4 other TNBC cell lines. (Fig 1b). Deguelin potently inhibited proliferation of MDA-MB-453 cells, with a GI₅₀ of 30 nM (Table 1). The GI₅₀ for deguelin was significantly higher in the other 4 cell lines, ranging from 12–17 μ M, indicating deguelin has 400 to 567-fold antiproliferative selectivity for the MDA-MB-453 cell line compared to cell lines representing other TNBC subtypes (Fig 1b). The total growth inhibitory concentration (TGI) of deguelin in the 5 cell lines was also determined. Deguelin had a TGI of 2 μ M in MDA-MB-453 cells (Fig. 1d, Table 1). This was significantly lower than the TGI in the other four cell lines, which ranged from 26–70 μ M. No significant differences among the other 4 TNBC cell lines for either measure of growth inhibition was observed. Next, we measured the selectivity of deguelin's cytotoxic activity. No significant difference in the LC₅₀, the concentration that causes 50% cell death, were observed among the cell lines (Fig. 1b).

To determine if this potent and highly selective activity of deguelin is specific to AR-expressing breast cancers, the activity of deguelin against another LAR cell line, SUM-185PE was evaluated. Deguelin potently inhibited proliferation and was cytotoxic to SUM-185PE cells, showing a biphasic concentration-response curve similar to that observed in MDA-MB-453 cells (Fig. 1b). The GI₅₀ of deguelin in SUM-185PE cells (61 nM) and the TGI (190 nM) were significantly lower in this cell line than the four cell lines modeling other TNBC subtypes (Fig. 1c, 1d). We next asked if deguelin has selectivity for other AR-expressing cells by evaluating its activity against a prostate cancer cell line. Interestingly, AR-dependent LNCAP cells showed intermediate sensitivity to deguelin, relative to MDA-MB-453 cells and the other four TNBC cell lines, with a GI₅₀ of 440 nM (Supplementary Fig. 1). This is consistent with AR being a target of deguelin, but indicates its expression does not result in maximal sensitivity. Based on these data, a concentration of 1 μ M deguelin was selected for subsequent mechanism of action studies because this concentration had nearly maximal effects in LAR cells, with minimal effects on the proliferation of the other TNBC cell lines.

The effects of 30 nM (GI₅₀) and 1 μ M deguelin on colony formation were evaluated in MDA-MB-453 cells (Figs. 1e, 1f). After only 8 h of treatment followed by drug washout, 1 μ M deguelin significantly inhibited MDA-MB-453 colony formation. Only one colony formed after treatment with deguelin, while 43 colonies formed after vehicle treatment (Fig. 1e). After 24 h of treatment, both 30 nM and 1 μ M deguelin significantly inhibited colony

formation compared to vehicle control. While 53 colonies formed after 24 h of vehicle treatment, only 15 colonies formed after treatment with 30 nM deguelin, and no colonies formed after treatment with 1 μ M deguelin (Fig. 1f). Collectively these results suggest that deguelin not only inhibited cell proliferation, but it was cytotoxic to MDA-MB-453 cells.

Effects of deguelin on PI3K/Akt/mTOR signaling and TNBC sensitivity to rapamycin

Published studies suggest the cytotoxic activity of deguelin in some cancer cells involves inhibition of signaling through the PI3K/Akt/mTORC1 pathway [41, 42]. To determine if deguelin inhibits PI3K/Akt/mTORC1 signaling, and if this could be responsible for its selective activity against LAR cells, deguelin-sensitive MDA-MB-453 cells and deguelin-resistant MDA-MB-231 cells were treated with vehicle or 1 μ M deguelin for 2–8 h and the phosphorylation of mTOR was evaluated (Fig. 2). Early time points after treatment were chosen so that initial signaling events altered by deguelin would be evaluated, rather than subsequent signaling changes due to initiation of cell death. In MDA-MB-453 cells, deguelin inhibited the phosphorylation of mTOR at S448 within 1 h of treatment, suggesting inhibition of PI3K/Akt signaling (Fig. 2a). To see if deguelin inhibited signaling downstream of mTOR complex 1 (mTORC1), the activation of the downstream effectors ribosomal protein S6 kinase (S6K), ribosomal protein S6 (RPS6), and 4E-BP1 were evaluated. Deguelin dramatically reduced phosphorylation of S6K and RPS6 in MDA-MB-453 cells as soon as 1 h after treatment (Fig. 2a). A reduction in phosphorylated RPS6 was also seen in SUM-185PE cells, although this was less than what was observed in MDA-MB-453 cells (Supplementary Fig. 2). In MDA-MB-453 cells, decreases in phosphorylated and total 4E-BP1 proteins were seen 2–8 h after treatment (Fig. 2a). In contrast to the effects in MDA-MB-453 cells, deguelin had no effect on the phosphorylation of S6K, RPS6 or 4E-BP1 in deguelin-resistant MDA-MB-231 cells (Fig. 2a). These results suggest that inhibition of mTORC1 signaling may be involved in the selective antiproliferative activity of deguelin against LAR cells. Interestingly, deguelin had no effect on the phosphorylation of Akt at S473, which is often phosphorylated by mTORC2 in response to mTORC1 inhibition [43].

MDA-MB-453 cells overexpress HER2 protein and depend on HER2-mediated signaling cascades, such as PI3K signaling, which could explain the sensitivity of MDA-MB-453 cells to deguelin. To determine if HER2-overexpressing cells in general are sensitive to deguelin, we measured deguelin's potency in HER2-overexpressing SK-BR-3 cells (Fig 1b). Deguelin had intermediate potency in SK-BR-3 cells compared to LAR cell lines and cells modeling other TNBC molecular subtypes, with a GI_{50} of 700 nM and TGI of 20 μ M (Table 1). These values were significantly higher than the GI_{50} and TGI in both LAR cell lines (Fig. 1c, 1d.), suggesting HER2 expression alone does not confer maximal deguelin sensitivity. Deguelin inhibited RPS6 phosphorylation in SK-BR-3 cells as well, further suggesting that inhibition of this signaling pathway is not the only predictor of deguelin sensitivity (Supplementary Fig 2).

We next sought to determine if mTORC1 inhibition is sufficient to reproduce the highly selective antiproliferative activity of deguelin in MDA-MB-453 cells by evaluating the antiproliferative effects of the mTORC1 inhibitor rapamycin in the same panel of breast cancer cell lines (Fig. 2b). While rapamycin showed the greatest maximal efficacy in MDA-

MB-453 and SUM-185PE cells, there were no significant differences in the GI_{50} values for rapamycin among MDA-MB-453, SUM-185PE, SK-BR-3, BT-549, HCC1937 or HCC70 cells (Fig. 2c). However, the GI_{50} of rapamycin was significantly higher in MDA-MB-231 cells than all of the other cell lines (Fig. 2c). To determine if upstream inhibition of mTORC1 produced selective effects similar to deguelin, the effects of the PI3K inhibitor LY294002 were also evaluated. LY294002 did not show selectivity for MDA-MB-453 cells compared to the other TNBC cell lines (results not shown). These results suggested that the selective activity of deguelin in LAR cells is not due to PI3K or mTORC1 inhibition alone.

Selectivity of 17-AAG for TNBC subtypes and its effects on mTORC1 signaling

Deguelin has been shown to bind Hsp90 and deplete Hsp90 client proteins, including Hif-1 α and Akt [44]. It is possible that the decrease in phosphorylated proteins observed after deguelin treatment is due to Hsp90 inhibition and resulting protein instability. To determine if LAR cells exhibit the same sensitivity to the Hsp90 inhibitor 17-AAG as they do to deguelin, the activities of 17-AAG were tested in the same cell line panel. The concentration-response curve of 17-AAG in MDA-MB-453 cells was reminiscent of deguelin's effects in this cell line. 17-AAG had the most potent antiproliferative effects and greatest efficacy in MDA-MB-453 and SUM-185PE cells compared to the other breast cancer cell lines (Fig. 3a). The GI_{50} for 17-AAG was significantly lower in the two LAR cell lines than the other five cell lines (Fig. 3b). However, the differences in GI_{50} for 17-AAG were notably less than the differences for deguelin between these cell lines. These results demonstrate that Hsp90 inhibition is not sufficient to reproduce the selective antiproliferative effects of deguelin against LAR cells.

To determine if Hsp90 inhibition could explain the reduction in RPS6 phosphorylation observed previously with deguelin, MDA-MB-453 cells were treated with vehicle, 1 μ M deguelin, or 50 nM 17-AAG (approximate TGI in MDA-MB-453 cells) and RPS6 phosphorylation was evaluated by immunoblotting at several time points. While deguelin reduced P-RPS6 levels in a time-dependent manner, 17-AAG had no effect on P-RPS6 levels 4–12 h after treatment (Fig. 3c). These results suggest that the deguelin-induced decrease in P-RPS6 and P-4E-BP1 in MDA-MB-453 cells is not a result of Hsp90 inhibition.

Effects of deguelin on androgen receptor (AR) protein in LAR cells

LAR TNBC cells are sensitive to Hsp90 inhibitors, but the molecular mechanisms are not clear. The mechanism likely involves destabilization of AR, which is a client protein of Hsp90, as it is well established that Hsp90 inhibitors induce proteasome-mediated degradation of Hsp90 client proteins. We next sought to determine if the highly selective effects of deguelin on LAR cells was due to an effect on AR by determining deguelin's effects on AR protein levels and cellular localization (Fig. 4). Deguelin significantly reduced AR protein levels in MDA-MB-453 cells after 18 h (Fig. 4a). Deguelin had little effect on AR abundance or localization after 2 h of treatment, but reduced cytoplasmic AR levels by approximately 40% relative to control cells after 18 h (Fig. 4a, b).

The ability of deguelin to inhibit the accumulation of AR in the nucleus of MDA-MB-453 cells in response to the synthetic AR agonist R1881 was also evaluated. MDA-MB-453 cells

express a mutant AR (AR-Q865H) with decreased sensitivity to dihydrotestosterone, but respond to androgen stimulation and are sensitive to antiandrogens *in vitro* [45, 46]. Treatment of androgen-starved cells with R1881 resulted in robust nuclear localization of AR. (Fig. 4c, d). Pretreatment with 1 μ M deguelin for 2 h or 18 h prior to R1881 stimulation resulted in a time-dependent decrease in nuclear AR, consistent with deguelin's effects on total AR levels. These results suggest deguelin depletes cellular AR levels in MDA-MB-453 cells and limits their ability to respond to androgen stimulation.

Effects of enzalutamide on MDA-MB-453 cells alone and in combination with rapamycin

Based on our findings that deguelin inhibited mTORC1 signaling, decreased AR levels, and prevented androgen-induced nuclear accumulation of AR, we hypothesized that the selectivity of deguelin emerged from combined inhibition of AR and mTOR. Therefore, the effects of a combination of an AR inhibitor and an mTOR inhibitor were tested to determine if they would have additive effects against MDA-MB-453 cells, thus explaining the high degree of selectivity of deguelin in this TNBC subtype. For these experiments, the potency of the second generation antiandrogen enzalutamide and the mTOR inhibitor rapamycin were evaluated in MDA-MB-453 cells. Enzalutamide inhibited growth and showed cytotoxic activity against MDA-MB-453 cells, with a GI₅₀ of 9.4 μ M and LC₅₀ of 84.7 μ M (Fig. 5a). Rapamycin more potently inhibited proliferation of MDA-MB-453 cells, with a GI₅₀ of 49.8 pM (Fig. 5a), but was purely antiproliferative, with no cytotoxic activity at concentrations up to 1 μ M. Collectively, these results demonstrate the distinctive sensitivities of MDA-MB-453 cells to pharmacological inhibition of AR or mTORC1.

We then determined whether enzalutamide and rapamycin have additive effects in MDA-MB-453 cells, by evaluating the ability of rapamycin to shift the concentration-response curve of enzalutamide. Rapamycin, at concentrations of 20 and 30 pM, increased the antiproliferative potency of enzalutamide, decreasing its GI₅₀ from 9.4 μ M to 7.6 μ M and 3.9 μ M, respectively (Fig. 5a). Rapamycin, at a concentration of 10 pM, did not change the GI₅₀ of enzalutamide (Fig. 5a). Interestingly, rapamycin only shifted the concentration-response curve at low concentrations of enzalutamide that produced antiproliferative effects. Rapamycin did not change the effects of higher, cytotoxic concentrations of enzalutamide, indicated by the near identical cytotoxic portions of their concentration-response curves. We next determined if the combination produced an effect equivalent to what would be expected if the two drugs were purely additive, or if they had synergistic activity. The combination of 20 pM rapamycin and 5 μ M enzalutamide inhibited MDA-MB-453 cell growth by approximately 40% relative to control, significantly more than either drug alone (Fig. 5b). The amount of growth inhibition produced by the combination was not significantly different than what would be expected if the drugs had additive effects. These results suggest enzalutamide and rapamycin have additive antiproliferative effects *in vitro*, with rapamycin contributing to the potent antiproliferative effects and enzalutamide contributing to the cytotoxic efficacy.

Antitumor activity of enzalutamide and rapamycin in a LAR xenograft model

We next evaluated the antitumor efficacy of each drug alone and in combination in a MDA-MB-453 xenograft model. We hypothesized that combining low doses of each drug would

have additive antitumor effects against this xenograft model and therefore a low dose of each drug was tested. Tumors were implanted into male mice based on studies demonstrating that MDA-MB-453 xenografts grow poorly in female mice without co-administration of androgens [46]. We first tested 6.25 mg/kg rapamycin, administered every other day, and 5 mg/kg enzalutamide administered daily (Supplementary Fig. S3). Enzalutamide treatment alone resulted in a moderate inhibition of tumor growth relative to vehicle-treated controls, but this was not statistically significant (Supplementary Fig. S3a, b). Treatment with rapamycin only or the combination of rapamycin/enzalutamide completely inhibited tumor growth, but there was no significant difference in final tumor volume between these two groups (Supplementary Fig. S3a, b). These doses did not cause a significant change in mouse body weight, suggesting that the combination is tolerable with minimal toxicity (Supplementary Fig. S3c). To confirm that rapamycin inhibited mTOR activity *in vivo*, tumor tissue samples from mice in each group were evaluated. Immunoblotting for P-RPS6 and total RPS6 showed that treatment with rapamycin alone or rapamycin/enzalutamide resulted in significantly lower levels of P-RPS6/total RPS6 compared to the vehicle control and enzalutamide groups (Supplementary Fig. S3d). No difference in P-RPS6/total RPS6 was observed between the rapamycin group and enzalutamide/rapamycin group.

A lower dose of rapamycin was subsequently evaluated to see if additive effects would be observed in combination with enzalutamide. Doses of 3.5 mg/kg rapamycin and 5 mg/kg enzalutamide were tested with the same dosing schedule (Fig. 6). In this experiment, enzalutamide alone inhibited tumor growth compared to vehicle controls, and resulted in a decrease in final tumor volume, as indicated by caliper measurements (Figs. 6a, b). Similar to the results of our first trial, treatment with rapamycin alone or enzalutamide/rapamycin completely inhibited tumor growth, with no difference in final tumor volume between these two groups (Figs. 6a, b). At trial conclusion, tumors were excised and weighed to determine their final mass. No significant difference in tumor mass was seen between the vehicle control group and enzalutamide group, in contrast to the caliper measurement data (Fig. 6c). Tumors from the rapamycin group and enzalutamide/rapamycin group had significantly lower mass than the vehicle and enzalutamide only groups, in agreement with our caliper measurements (Fig. 6c). Immunoblotting of tumor lysates from this trial showed that rapamycin alone or rapamycin/enzalutamide significantly inhibited phosphorylation of RPS6 compared to the vehicle and enzalutamide groups (Figs. 6d, e). There was no difference in P-RPS6/total RPS6 between the rapamycin group and enzalutamide/rapamycin group, similar to the results seen with tumor growth (Figs. 6d, e). Collectively, these results indicate that rapamycin has potent antitumor efficacy in this xenograft model, and suggests that enzalutamide may have antitumor activity at higher doses than those tested for this study. Further studies evaluating lower doses of rapamycin will be necessary to determine if rapamycin and enzalutamide have additive effects *in vivo*.

Discussion

The goal of this project is to identify new agents and targets for the treatment of TNBC subtypes. Deguelin is not a new compound and its activity, with potency in the micromolar range, has been previously reported against a range of cancer cell lines. The high potency of deguelin in LAR cells provided the opportunity to identify targets responsible its activity.

Deguelin, as a member of the rotenone family of toxins, does not have potential for drug development. These results suggest that deguelin has high potency and efficacy in LAR cell lines due to its ability to inhibit multiple targets that drive the LAR subtype of TNBC.

Our results show that deguelin inhibits both mTORC1 signaling and decreases AR levels in AR-expressing TNBC cells and thereby selectively inhibits proliferation of LAR cell line models. The same concentration that inhibits both of these pathways in the sensitive LAR cells has no effect on mTOR signaling in MDA-MB-231 cells, representing another subtype of TNBC. Interestingly, Lehmann and Bauer *et al.* demonstrated that LAR TNBC cell lines are not only sensitive to antiandrogens, but also Hsp90 inhibitors [27]. It is reasonable to speculate that the sensitivity of LAR TNBC cells to Hsp90 inhibitors may be a result of AR protein proteolysis, since Hsp90 inhibitors such as 17-AAG induce proteasome-mediated degradation of Hsp90 clients. Deguelin has been shown by many groups to inhibit the activity of Hsp90 by preventing ATP binding and the natural cycling of this chaperone protein [44, 47]. It is likely that this activity of deguelin is a major contributor to its cellular mechanism of action in LAR cells, as our data indicate that deguelin reduces AR levels. However, neither Hsp90 nor mTOR inhibition alone can explain the high degree of selectivity observed with deguelin *in vitro*, suggesting both mechanisms are involved.

There is an increasing body of evidence suggesting that AR plays a major role in multiple subtypes of breast cancer [48]. It has been reported that ~80% of breast cancers express AR [49], and several groups have reported the sensitivity of TNBC cell lines and xenografts to the antiandrogens bicalutamide and enzalutamide [29, 46]. Interest in inhibition of AR as a treatment for AR-expressing breast cancers led to the initiation of clinical trials evaluating the efficacy of AR inhibitors in breast cancer patients. A clinical trial of the first generation antiandrogen bicalutamide in AR+ breast cancer patients began in 2007 [50], and the first trial evaluating the safety and efficacy of enzalutamide in patients with advanced, AR+ TNBC was initiated in 2013. Despite a growing body of literature demonstrating the dependence of multiple molecular subtypes of TNBC on AR, little is known about the activity of enzalutamide against this subtype.

In addition to AR expression, mutations or overexpression of *PIK3CA* are found in 40% of AR-positive TNBC cases [29]. Thus, targeting the PI3K/mTOR pathways in the LAR subtype is a rational approach. Many different PI3K inhibitors with selectivity for different PI3K isoforms, as well as mTOR inhibitors, have been evaluated in clinical trials. A phase II trial evaluating the mTOR inhibitor everolimus as monotherapy in metastatic breast cancer patients demonstrated activity in only 12% of patients receiving daily treatment, but no effect in patients receiving weekly treatment, suggesting dosing schedule is important for the antitumor efficacy [51]. Recently, a clinical trial was initiated to evaluate the efficacy of the PI3K inhibitor taselisib in combination with enzalutamide in metastatic, AR+ TNBC. This will be the first trial to evaluate the combination of enzalutamide and a PI3K inhibitor in breast cancer patients, and will provide an initial indication whether this is an effective treatment strategy. It is worth noting that we observed selectivity for MDA-MB-453 cells only with rapamycin and not the PI3K inhibitor LY294002, suggesting that the point of inhibition in the PI3K/Akt/mTOR signaling pathway may be important for selectivity. Our results show that inhibition of mTORC1 signaling, particularly inhibition of RPS6

phosphorylation, produces selective growth inhibition in AR-expressing breast cancer cells. This supports findings by Elkabets, Vora, Juric and colleagues that patient response to the PI3K p110 α inhibitor BYL719 was associated with inhibition of RPS6 phosphorylation [52].

Our results suggest that combined inhibition of mTOR signaling and AR might be effective for treating AR-expressing breast cancer. However, further studies are needed to determine how to optimize combinations of drugs targeting these pathways. In our studies, the combination of low doses of both rapamycin and enzalutamide were tolerable, warranting further investigation into these drugs. Significantly higher doses of enzalutamide, up to 50 mg/kg/day, have been evaluated by other groups in mouse models with no noticeable toxicity [48]. Based on this it is possible that higher dose of enzalutamide could be utilized, with no adverse effects on tolerability, to produce additive antitumor effects and tumor regression. It is possible that doses of rapamycin and enzalutamide that can induce regression of larger, established tumors will be identified. This would be important for treating patients with advanced disease, but additional experiments will be needed to determine how to optimize this drug combination. The advantage of using low, highly tolerable doses of rapamycin and enzalutamide is that it might be possible to combine them with a cytotoxic agent to improve antitumor efficacy.

There is clearly a need to develop better, less toxic therapies to treat breast cancer. This can only be achieved by a better understanding of the molecular drivers of these heterogeneous diseases. Recently, Purrington *et al* independently identified six TNBC subtypes through gene expression profiling which included genes associated with histopathologic features of TNBC [53]. These subtypes are similar to the subtypes identified by Lehmann and Bauer *et al*, including one defined by a luminal signature and high AR expression. By screening natural product libraries for selective activity against TNBC molecular subtypes, we were able to identify molecular targets with therapeutic potential for treating subtypes of this disease. Natural products are often capable of affecting multiple molecular targets, which will be particularly valuable for identifying the oncogenic signaling pathways driving TNBC molecular subtypes. Additionally, the unique chemical properties of natural products make it likely that screening natural product libraries will identify molecules affecting a wide range of cellular targets. Our results provide rationale for further investigations into the role of Hsp90, AR and mTORC1 in AR-expressing TNBC, as well as studies evaluating the efficacy drugs that target these proteins. These results also demonstrate that screening natural products from diverse sources has excellent potential to identify compounds and molecular pathways with therapeutic importance for treating molecular subtypes of TNBC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Deguelin selectively inhibits proliferation of AR-expressing TNBC cells and inhibits colony formation after drug washout. **a** Structure of deguelin. **b** Sulforhodamine B assay concentration-response curves for growth inhibition of six TNBC cell lines and HER2+ SK-BR-3 cells after treatment with deguelin for 48h. Results represent mean $\pm$ SE for $n = 3-7$ independent experiments, with each concentration tested in triplicate. **c, d** Comparison of GI_{50} and TGI values for deguelin in seven breast cancer cell lines representing different TNBC molecular subtypes and HER2+ cancers; $n = 3-7$ independent experiments. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to MDA-MB-453; # $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$ compared to SUM-185PE; one-way ANOVA with Tukey's post-hoc test. **e, f** Inhibition of MDA-MB-453 colony formation after treatment with deguelin for 8h

or 48h. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to vehicle; one-way ANOVA with Tukey's post-hoc test

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Fig. 2.

Inhibition of mTORC1 signaling by deguelin and TNBC cell line sensitivity to rapamycin. **a** Immunoblotting of MDA-MB-453 (left) and MDA-MB-231 (right) whole-cell lysates for downstream targets of mTORC1 signaling and assessment of their phosphorylation status. Cells were treated with vehicle (DMSO) or 1 μ M deguelin for increasing amounts of time, as indicated, then lysed and the soluble proteins separated by SDS-PAGE. Results shown are representative of at least two independent experiments. **b** Sulforhodamine B assay concentration-response curves for rapamycin in breast cancer cell lines representing five molecular subtypes of TNBC and HER2+ cancers; Results represent mean $\pm$ SE for $n = 3-6$ independent experiments, with each concentration tested in triplicate. **c** Comparison of GI_{50} value for rapamycin in each cell line; $n = 3-6$ independent experiments, with each concentration tested in triplicate. **** $p < 0.0001$ compared to MDA-MB-231; one-way ANOVA with Tukey's post-hoc test

Fig. 3. Effects of Hsp90 inhibitor 17-AAG on TNBC cell proliferation and mTORC1 signaling. **a** SRB assay concentration-response curves for 17-AAG in breast cancer cell lines representing 5 molecular subtypes of TNBC and HER+ cancers; Results represent mean $\pm$ SE for $n = 4-7$ independent experiments, with each concentration tested in triplicate. **b** Comparison of GI_{50} values for 17-AAG in each cell line; $n = 4-7$ independent experiments, with each concentration tested in triplicate. *** $p < 0.001$, **** $p < 0.0001$ compared to MDA-MB-453; ### $p < 0.001$, #### $p < 0.0001$ compared to SUM-185PE; one-way ANOVA with Tukey's post-hoc test. **c** Immunoblotting of MDA-MB-453 whole-cell lysates for P-RPS6 and RPS6. Cells were treated with vehicle (DMSO), 1 μ M deguelin or 50 nM 17-AAG for increasing amounts of time, as indicated, then lysed and the soluble extracts separated by SDS-PAGE. Results shown are representative of at least two independent experiments

Fig. 4. Deguelin decreases androgen receptor abundance and inhibits nuclear accumulation in response to R1881. **a** Representative immunoblot of AR after 1 μ M deguelin treatment for 18 h. **b** Relative quantification of immunoblotting signals. **c** Evaluation of androgen-stimulated AR nuclear accumulation after deguelin treatment. MDA-MB-453 cells were cultured in media containing 10% charcoal/dextran-stripped serum for 3 days and then pretreated with vehicle for 18h or deguelin (1 μ M) for 2h or 18h. Cells were subsequently treated with 1 nM R1881 for 2h and then extracted to obtain cytosolic and nuclear fractions, which were separated by SDS-PAGE and detected by immunoblotting. **d** Relative quantification of AR abundance in nuclear fractions

Fig. 5.

Enzalutamide and rapamycin have additive effects on MDA-MB-453 cell proliferation. **a** SRB assay concentration-response curves for enzalutamide, rapamycin and the combination of enzalutamide with a single concentration of rapamycin, in MDA-MB-453 cells. Results represent mean $\pm$ SE for $n = 3$ independent experiments, with each concentration tested in triplicate. **b** Quantification of growth inhibition produced by enzalutamide (5 μ M), rapamycin (20 pM) and the combination. * $p < 0.05$, ** $p < 0.01$; results represent mean $\pm$ SE for $n = 3$ independent experiments

Fig. 6.

Enzalutamide and rapamycin inhibit growth of MDA-MB-453 xenografts. **a** Caliper measurements of tumor volumes taken twice weekly over course of treatment. Results represent mean $\pm$ SD for $n = 8-10$ tumors in each group (8 in enzalutamide/vehicle group; see methods). **b** Individual tumor volumes on final day of treatment. Results represent mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; one-way ANOVA with Tukey's post-hoc test. **c** Individual masses of excised tumors after flash-freezing at conclusion of experiment. Results represent mean $\pm$ SD. *** $p < 0.001$, **** $p < 0.0001$; one-way ANOVA with Tukey's post-hoc test. **d** Immunoblotting of tumor lysates (3 mice per group) for phosphorylation of S6 ribosomal protein. **e** Quantification of immunoblotting signals **** $p < 0.0001$; one-way ANOVA with Tukey's post-hoc test

Table 1

Antiproliferative potency of deguelin in seven cell lines modeling five different molecular subtypes of TNBC and HER2+ breast cancer

Cell Line	Subtype	GI ₅₀ (μM)	TGI (μM)
MDA-MB-453	LAR	0.03 ± 0.02	2 ± 4
SUM-185PE	LAR	0.061 ± 0.007	0.19 ± 0.04
SK-BR-3	HER2+	0.7 ± 0.2	20 ± 20
BT-549	ML	17 ± 9	30 ± 10
HCC70	BL2	16 ± 4	26 ± 8
MDA-MB-231	MSL	15 ± 5	30 ± 6
HCC1937	BL1	12 ± 7	70 ± 20

GI₅₀, concentration causing 50% inhibition of proliferation; TGI, total growth inhibitory concentration. Results represent mean ± SD of n = 3–7 experiments.