

Antiandrogenic activity and bioavailability of magnolol analogs – A potential for prostate cancer therapeutics

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ARTICLE INFO

Keywords:
Magnolol
Prostate
Cancer
Antiandrogenic

ABSTRACT

Background: Prostate cancer is the second most common form of cancer in men worldwide and there is a great need for novel treatment strategies, especially for castrate-resistant prostate cancers where the proliferation of the cancer cells is stimulated by androgens produced in the adrenal cortex and the cancer cells.

Purpose: In this study, we have investigated the antiandrogenic properties of magnolol and ten synthetic analogs *in vitro*.

Study design and methods: The compounds were evaluated for cytotoxicity, antiandrogenic receptor activity, binding to the androgen receptor, effects on the production of Prostate-specific antigen (PSA), and potential to pass over a tight layer of Caco-2 cells mimicking gastrointestinal absorption.

Results: We found that almost all investigated compounds were antiandrogenic in an androgen receptor reporter gene assay, with IC₅₀ values ranging from 7 to 86 μM. Magnolol itself had the highest antiandrogenic potency. Five of the compounds were then evaluated for their binding to the androgen receptor and three of these compounds were found to bind to the receptor. These five compounds were also evaluated for their effect on the PSA production and four were found to decrease PSA production at non-cytotoxic concentrations. The antiandrogenic activity after passage through a layer of Caco-2 cells, mimicking gastrointestinal absorption, was also evaluated for three of the compounds. All three compounds were found to have the capacity to be transported from the apical to the basolateral side of the Caco-2 cell layer and exert antiandrogenic effects after the transport.

Conclusion: In conclusion, this study shows that magnolol and analogs have antiandrogenic effects *in vitro* and that selected analogs can pass over a tight layer of Caco-2 cells, indicating a potential for good bioavailability after oral administration. These magnolol analogs thereby constitute an interesting group of compounds worthy of further evaluation as potential anti-prostate cancer therapeutics.

Introduction

Prostate cancer is the second most common form of cancer in men worldwide according to the World Health Organization with more than 1.4 million new cases in 2020, causing 375 000 deaths per year (GLOBOCAN 2020). Prostate cancer growth is dependent on androgens, mainly produced in the testes. The common initial treatment of prostate cancer is androgen-deprivation therapy, accomplished through surgical or chemical castration, stopping production of testosterone in the testes (Litwin and Tan 2017). However, after some time this treatment fails, and castration-resistant prostate cancer develops in one third of the patients. At this stage, the prostate cancer cells are stimulated by

androgens, synthesized in the adrenal cortex and in the cancer cells (Barnard et al., 2020). Treatment of castrate-resistant prostate cancer includes inhibition of androgen synthesis (abiraterone) and inhibition of androgen receptor activity (enzalutamide, apalutamide and darolutamide) and a combination of these (Chandrasekar et al., 2015; Mori et al., 2020; Posdizich et al., 2023). Plant-derived bioactive constituents, especially phenolic compounds, are demonstrated to have promising properties for prevention and treatment of prostate cancer (Hao et al., 2022; Salehi et al., 2019).

Magnolol (IUPAC name 2-(2-hydroxy-5-prop-2enylphenyl)-4-prop-2-enylphenol) is a dimeric neolignan (bisphenol neolignan), found in the roots and bark of the magnolia tree, *Magnolia officinalis*. Lignans are

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<https://doi.org/10.1016/j.phyplu.2023.100485>

Available online 1 September 2023

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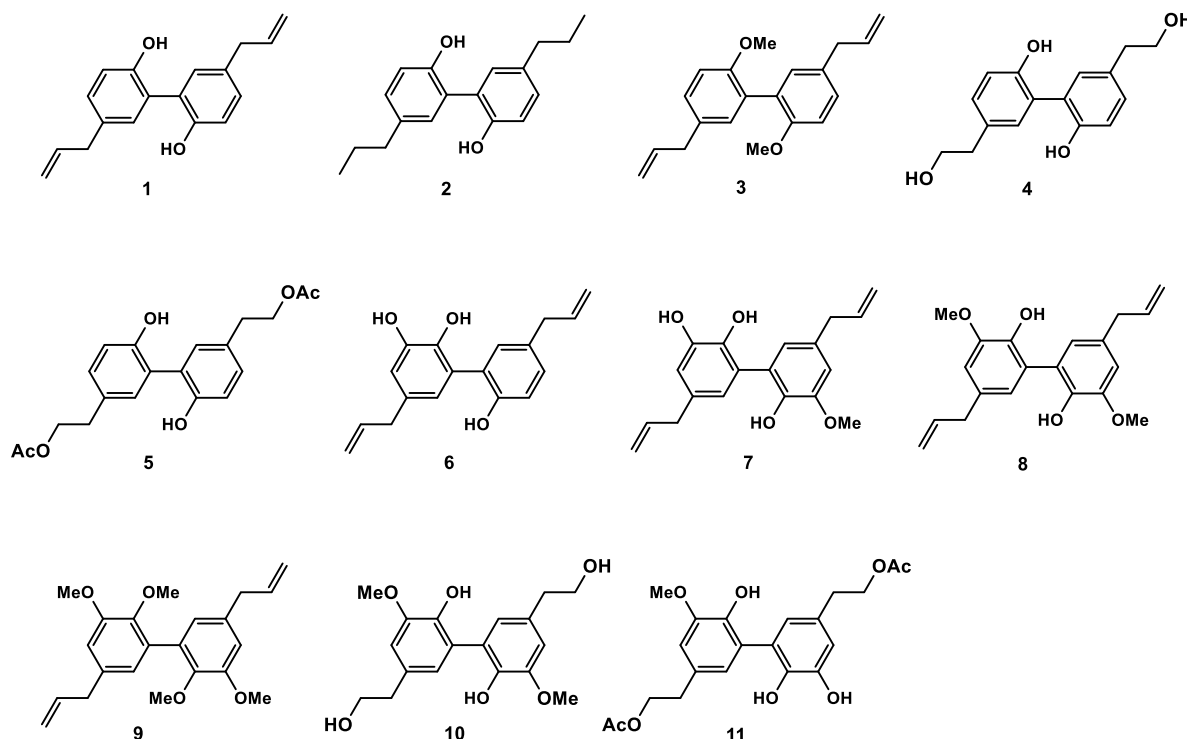


Fig. 1. Chemical structures of magnolol (1) and the 10 analogs used in this study.

polyphenols, present in various plants as secondary metabolites. Magnolol has a wide range of biological activities including anti-oxidative, anti-inflammatory, anti-microbial, antiviral, neuroprotection, cardiovascular protection and metabolism regulation (reviewed by Zhang et al. (2019); (Lin et al., 2021)). Magnolol has been proposed to be used in chemoprevention of various cancer forms, via effects on cell growth, apoptosis, angiogenesis and metastasis (Chen et al., 2019; Ranaware et al., 2018; Wang et al., 2022).

Magnolol affects human prostate cancer cells *in vitro* by different mechanisms: abolished cell proliferation by arresting cells at G0/G1 phase and via P53/P21 activation (Huang et al., 2017), apoptosis via inhibition of EGFR and AKT signaling (Lee et al., 2009), suppressed metastatic properties by downregulation of MMP-2 and MMP-9 (Hwang and Park 2010) and restrained cell growth by downregulation of IGF-1 (McKeown and Hurta 2015). However, the effects of magnolol on androgen receptor activity have to our knowledge not been investigated previously.

The clinical application of magnolol is however limited due to a low bioavailability and a rapid metabolism (Zhang et al., 2019). With the aim to improve the biological properties and increase kinetic stability, magnolol analogs have been synthesized and tested for various bioactivities. Pulvirenti et al. (2017) studied the possible utilization of magnolol analogs as antidiabetic drugs and reported a high inhibition of α -glucosidase *in vitro*. Baschieri et al. (2017) investigated the structure-activity relationships of synthetic magnolol analogs concerning the anti-oxidant activity. Methylated and acetylated magnolol were reported to have improved cutaneous anti-inflammatory properties compared to the parent compound magnolol (Lin et al., 2016). Further, bisphenol neolignans related to magnolol have shown anti-inflammatory (Lee et al., 2012), antimicrobial (Jada et al., 2012), antitumor (Di Micco et al., 2018; Lin et al., 2012) and neuroprotective activity as well as modulation of GABA receptors (Fuchs et al., 2014).

We have investigated the *in vitro* effects of magnolol and 10 synthetic analogs (Fig. 1) on antiandrogenic receptor activity and androgen receptor binding. Prostate-specific antigen (PSA) expression was determined as an indicator of downstream androgen receptor signaling.

Furthermore, to assess the potential activity after peroral administration of the test compounds, we studied the androgen receptor activity of compounds under study after transport through a Caco-2 cell layer, mimicking the gastrointestinal absorption.

Materials and methods

Test compounds

Magnolol (1) was purchased from TCI Europe (Milan, Italy). The magnolol analogs employed in this study are reported in Fig. 1. Compounds 4, 5, 6, 7, 8, 10 and 11 were synthesized as previously reported by some of us (Pulvirenti et al., 2017).

Compound 3 was obtained by methylation of 1, according to the following procedure: magnolol (1, 20.0 mg, 0.075 mmol) was dissolved in dry acetone (7.7 mL), and then K_2CO_3 (20.7 mg, 0.15 mol) and CH_3I (7.6 μ L, 0.15 mmol) were added. The solution was stirred at reflux for 48 h and then quenched. After evaporation of the solvent under vacuum, the residue was submitted to flash chromatography on DIOL Silica-gel, eluting with *n*-hexane: $CHCl_3$ (from 100:0 to 30:60) to give the permethylated derivative (3). Its MS and NMR data are in agreement with those previously reported in the literature (Lin et al., 2013). Compound 2 was prepared by hydrogenation of 1 (30 mg, 0.11 mmol). The reaction was carried out in absolute EtOH (8 mL) employing Pd/CaCO₃ (10% w/w; 8 mg) as a catalyst. The reaction flask was filled with H₂ (1 atm) and stirred at room temperature for 24 h. The catalyst was removed by filtration on Celite 545. The expected product 2 was obtained with quantitative yield without further purification. Its spectroscopic data are in agreement with those previously reported (Kong et al., 2005).

The synthesis of compound 9 was performed in two steps. Firstly, an enzymatic dimerization of eugenol was carried out with an HRP solution and H₂O₂ according to a procedure previously reported by some of us to obtain 5,5'-diallyl-3,3'-dimethoxy-[1,1'-biphenyl]-2,2'-diol (Pulvirenti et al., 2017). The purified product (17 mg; 0.052 mmol) was dissolved in dry acetone (1 mL) and treated with anhydrous K₂CO₃ (41 mg; 0.312 mmol) under stirring at room temperature for 10 min. Then, CH_3I (19

μL) was added to the reaction flask and the mixture was refluxed for 24 h. After 5 h, another aliquot of CH_3I (19 μL) was added. The reaction mixture was taken to dryness under vacuum, and the residue was submitted to flash chromatography with DIOL Silica-gel, eluting with *n*-hexane: CHCl_3 (from 100:0 to 50:50). The expected product **9**, R_f 0.6 (ethyl acetate:*n*-hexane, 30:70), was obtained with 60% of yield (10.2 mg). Its NMR data are in agreement with those previously reported (Lievot et al., 2015).

All test compounds were dissolved in dimethyl sulfoxide (DMSO) purchased from Sigma-Aldrich.

Cell culture

Human prostate LNCaP cells were cultured in RPMI 1640 medium (Gibco) supplemented with 2 mM L-glutamine (Gibco), 10% fetal bovine serum (Gibco), 1 mM sodium pyruvate (Gibco), and 100 U mL^{-1} penicillin and 100 $\mu\text{g mL}^{-1}$ streptomycin (Gibco).

A Chinese Hamster Ovary (CHO) cell line stably transfected with an AR responsive luciferase plasmid and an expression vector for the human androgen receptor (AR-EcoScreen cell line) was obtained from the Japanese Collection of Research Biosources Cell Bank and used for the AR reporter gene assay. The cells were cultured in DMEM F12 medium without phenol red (Sigma) supplemented with 5% FBS (Gibco), 2 mM L-glutamine (Lonza, Basel, Switzerland), 100 U mL^{-1} penicillin, 100 $\mu\text{g mL}^{-1}$ streptomycin, (Lonza), 100 $\mu\text{g mL}^{-1}$ Hygromycin B (InvivoGen, USA), and 200 $\mu\text{g mL}^{-1}$ Zeocin (Invitrogen, CA, USA). Experimental medium consisted of DMEM F12 (Sigma) medium supplemented with 5% dextran-charcoal treated fetal bovine serum (Thermo Scientific), 4 mM L-glutamine (Gibco, Thermofisher Scientific), Penicillin/Streptomycin with a final concentration of 100 U mL^{-1} penicillin, 100 $\mu\text{g mL}^{-1}$ streptomycin, (Lonza).

The human colon carcinoma cell line (Caco-2) was cultured in Caco-2 medium with Dulbecco's Modified Eagle medium DMEM F12, 10% FBS (Gibco), 100 U mL^{-1} penicillin, 100 $\mu\text{g mL}^{-1}$ streptomycin, 2 mM L-glutamine and 1% non-essential amino acids (Gibco).

All cells were cultured in a humidified incubator maintained at 37 °C in an atmosphere of 5% CO_2 . The medium was exchanged twice a week. For passaging, cells were detached with Trypsin-EDTA.

In all experiments, except the Caco-2 transwell experiments described in Section 2.6, vehicle control consisted of 1% DMSO, equivalent to the DMSO concentration in the test compound solutions.

Cell viability assay

Cell viability was assayed in AR-EcoScreen cells and LNCaP cells using the MTS assay. Cells were seeded in 96 well plates with a density of 1×10^4 cells per well and incubated for 24 h. AR-EcoScreen cells were exposed to compounds **1** – **11** in a three-fold dilution series with a concentration range from 1.2 to 100 μM . LNCaP cells were exposed to compounds **1**, **4**, **6**, **7**, and **11** at 33 and 100 μM , which were the compounds and the concentrations used for PSA analysis in LNCaP cells. Cell viability was measured after 24 h using the CellTiter 96 Aqueous One Solution Proliferation Assay System (Promega) in accordance with the protocol of the manufacturer. The absorbance was measured using a Wallac Victor2 1420 microplate reader (PerkinElmer) or Tecan Infinite®M1000 Pro plate reader (TECAN, Austria GmbH, Austria). Effects of test compounds on viability were compared to vehicle control. Exposure resulting in a cell viability of <80% as compared to the vehicle control was considered cytotoxic.

Antiandrogen receptor activity

Antiandrogen receptor activity was assayed using the AR-EcoScreen cell line. All experiments were conducted in white-walled, clear-bottomed, 384 well plates (Corning, NY, USA) over a three day period with cell seeding on day 1, cell treatment with magnolol (**1**), analogs **2** –

11 and standards on day 2, and luciferase measurement on day 3. Cells were seeded in a density of 4 000 cells per well. To assay AR antagonistic activity, AR activity was induced by 500 pM dihydrotestosterone (DHT), which was added to the cells together with the test compounds. Hydroxyflutamide (OHF) (Sigma-Aldrich, USA) was used as a positive control for AR antagonism and tested in 6 concentrations in the range of 10^{-5} M to 10^{-10} M.

At experiment termination, cells were lysed with passive lysis buffer (PLB) (Promega), 10 $\mu\text{L well}^{-1}$, for 15 min in 384 well plates. Luciferase activity was measured using the Luciferase® Reporter Assay System (Promega) according to the manufacturer's instructions. Luminescence was measured on a Tecan plate reader (TECAN, Austria GmbH, Austria) with an automatic injection syringe. The injection volume for the Firefly luciferase reagent was 10 μL per well. Luminescence measurement was conducted over a 5 s period, 2 s after reagent was automatically injected with Firefly luciferase reagent. White adhesive sealing film was attached to plate bottom before measurement. Receptor activity was normalized to vehicle control and set to 1. The normalized receptor activity for each treatment group was plotted against the concentration of the compound and a curve fitting was performed using GraphPad Prism 7.

Androgen receptor binding assay

PolarScreen Androgen Receptor Competitor Assay (Thermo Fisher) was used, in accordance with the manufacturer's recommendations, to study the binding potential of magnolol and analogs to the androgen receptor. In this cell-free system, the androgen receptor binding of test compounds can be studied using purified AR protein and a tight-binding selective fluorescent androgen receptor ligand; Fluormone Tracer. In the absence of other androgen receptor ligands, Fluormone Tracer binds to the ligand binding domain of androgen receptor and the complex exerts a high fluorescence polarization value. In the presence of increasing concentrations of other androgen receptor ligands, the Fluormone Tracer will be displaced from the androgen receptor ligand binding domain and the displaced tracer will have a significantly lower fluorescence polarization value. The compounds **1**, **4**, **6**, **7** and **11** were analyzed for androgen receptor binding properties in a 16-point, 3-fold dilution series with a concentration range from 63 pM to 100 μM . The known androgen receptor ligand dihydrotestosterone (DHT) was used as a positive control of Fluormone Tracer displacement and analyzed in a 16-point, 3-fold dilution series with a concentration range from 3.5 pM to 50 μM . The assay was performed in black low volume 384 well plates (Corning) and the fluorescence polarization value for each sample was analyzed using an Infinite M16000 plate reader (Tecan). As the assay maximum control we used a sample without competing ligand. This sample represents the highest fluorescence polarization value possible, as the Fluormone Tracer is completely bound to the androgen receptor. As the assay minimum control, we used a sample with 50 μM DHT, to represent the lowest possible polarization value when all Fluormone Tracer is displaced from the androgen receptor. The polarization value for each treatment group was plotted against the concentration of the compound and a curve fitting was performed using GraphPad Prism 7.

Prostate-specific antigen (PSA)-expression assay

Prostate-specific antigen (PSA) is a protein, which expression is regulated by ligand-activated androgen receptor. Therefore, it is used as a downstream biomarker of androgen receptor signaling in prostate cells. To investigate if selected magnolol analogs alter androgen receptor signaling in prostate cells, LNCaP cells were cultured as described above. LNCaP cells were seeded in 96 well plates with a density of 2×10^4 cells and incubated for 48 h. Following the incubation, the cell culture medium was changed and the cells exposed to magnolol and analogs (**1**, **4**, **6**, **7**, **11**) in concentrations of 33 or 100 μM for 24 h. The cell culture medium was collected and the PSA level was analyzed using a PSA specific ELISA kit from Demeditec (kit DE3719). The PSA level was

AR-EcoScreen cells

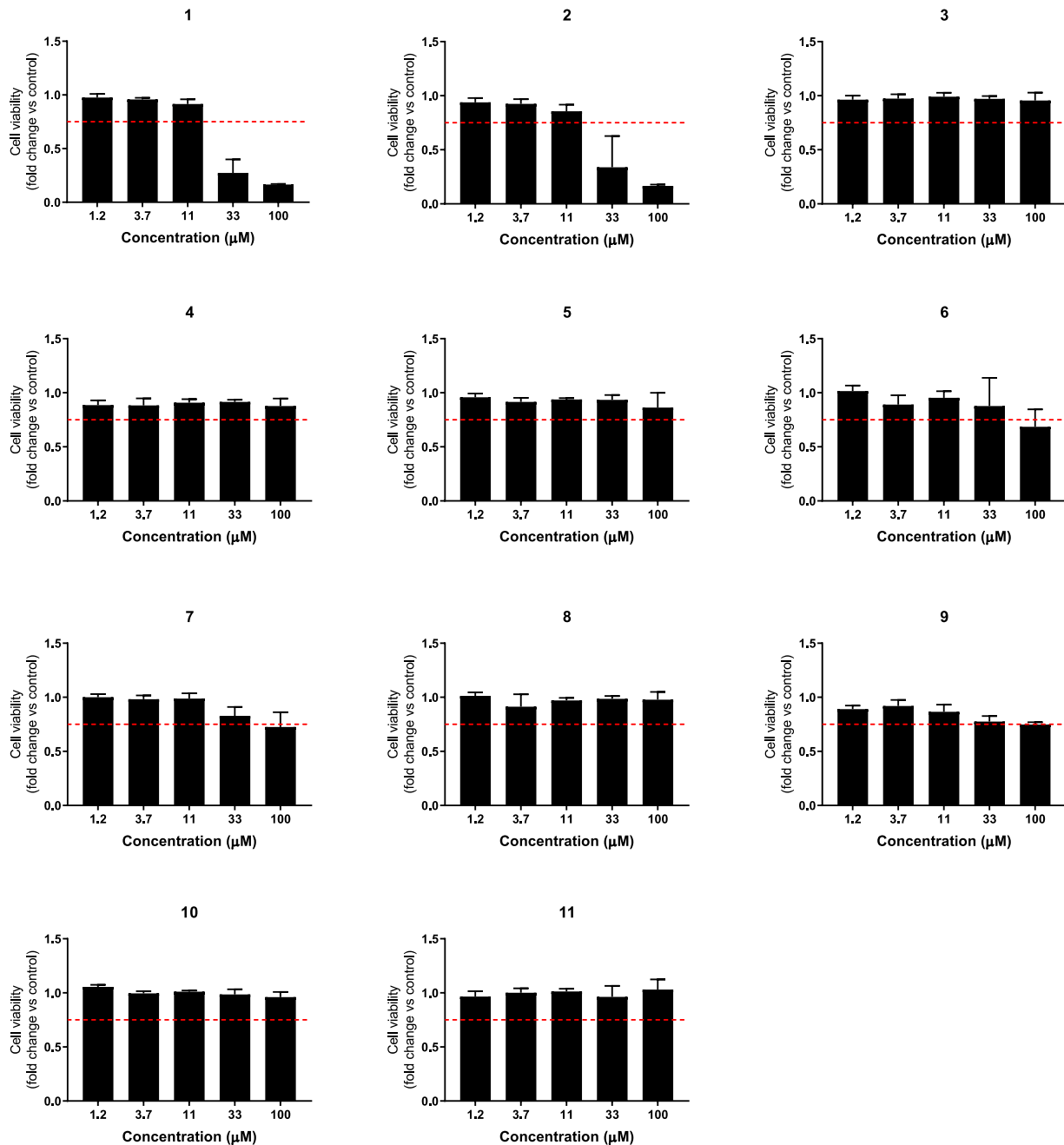


Fig. 2. Cell viability of AR-EcoScreen cells following 24 h exposure to magnolol (1) and the ten analogs. For identification of analogs, see Fig. 1. A cell viability of <80% compared to the vehicle control was defined as cytotoxicity, which is marked by the dotted line. Mean ± SD; n = 4.

compared to a vehicle treated control.

Transwell assay with Caco-2 cell monolayer in upper and AR-EcoScreen cells in lower compartments

To investigate the potential absorption of magnolol and analogs after administration *per os*, we used a transwell model where human colorectal carcinoma Caco-2 cells, which is an experimental model of human absorptive enterocytes, cultured as a tight layer in the upper compartment and the AR-EcoScreen cells in the lower compartment of the well.

Trans epithelial electrical resistance (TEER)

TEER is a measurement of ion flow through the cell monolayer and is a marker for the integrity of the monolayer. TEER across Caco-2 cell monolayer was measured with an epithelial voltometer EVOM (World Precision Instruments) and STX2 electrode. TEER was determined as ohm (Ω) per filter insert, which was later calculated to $\Omega \text{ cm}^2$. TEER was measured before and at the end of the experiment and the results expressed as $\text{TEER}(\%) = (\text{TEER before exposure} / \text{TEER at the end of exposure}) \times 100$.

Cell transport studies

Caco-2 cells were seeded on the apical filters of the Transwell®

LNCaP cells

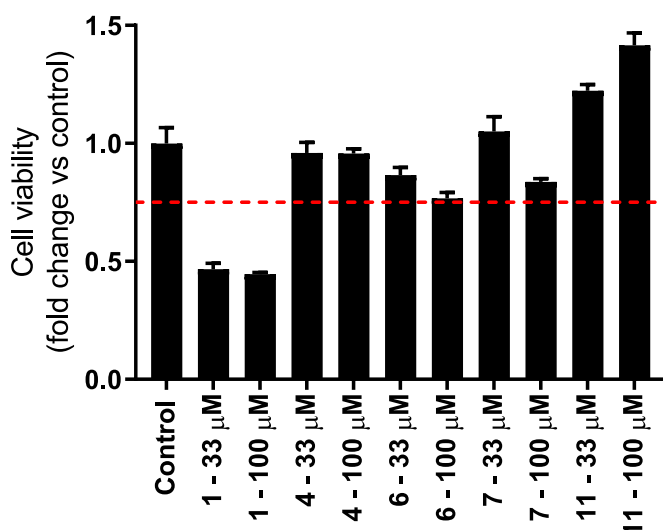


Fig. 3. Cell viability of LNCaP cells following 24 h exposure to magnolol (1) and four analogs. For identification of analogs, see Fig. 1. A cell viability of <80% compared to the vehicle control was defined as cytotoxicity, which is marked by the dotted line. Mean \pm SD; $n = 4$.

inserts in 12-well plates (Corning) at a density of 6×10^5 cells per insert. Cells were grown in the Caco-2 medium and medium was exchanged twice a week in both the apical (0.5 ml) and the basolateral (1.5 ml) chambers. Experiments were performed after cells were grown for 21–25 days. Before experiments, the integrity of the cell monolayer was assessed by transepithelial electrical resistance (TEER) and found to be $>1000 \Omega \text{ cm}^2$.

After 21–25 days, when the Caco-2 cells had formed a monolayer with a TEER value $>1000 \Omega \text{ cm}^2$, AR-EcoScreen cells were plated in the lower compartment of a Transwell plate at a density of 1.8×10^5 cells per well in AR-EcoScreen experimental medium. The cell culture medium in the upper transwell was also exchanged to AR-EcoScreen experimental medium and the cells were incubated for 24 h. Following incubation, TEER values $>1000 \Omega \text{ cm}^2$ were confirmed for the Caco-2 cell monolayer in all wells. Magnolol and analogs 4, 6, 7 and 11 were added to the apical chamber of the Transwell plate. Dihydrotestosterone (DHT) (Sigma-Aldrich, USA) in a concentration of 500 pM was added together with the samples and standards. DMSO (0.4%) with and without DHT were used as negative controls and 1 μM hydroxyflutamide (OHF) (Sigma-Aldrich, USA), the prostate cancer drugs abiraterone at 1 and 10 μM (Selleck Chemicals) and enzalutamide at 10 μM (Selleck Chemicals) were used as positive controls.

Samples were incubated for 4 h with inserts and TEER values were recorded at 10, 30, 60, 120, and 240 min. After 4 h, the apical inserts were discarded and the plate was incubated for an additional 20 h.

At experiment termination, AR-EcoScreen cells from the lower compartment were lysed with passive lysis buffer (PLB) (Promega) for 15–20 min. Cell lysate, 10 μL well per well, was transferred from the 12-well to a 384-well plate and luciferase activity was measured using the Luciferase® Reporter Assay System (Promega) according to the manufacturer's instructions. Luminescence was measured on a Tecan Infinite®M1000 Pro plate reader (TECAN, Austria GmbH, Austria) with an automatic injection syringe. The injection volume for the Firefly luciferase reagent was 10 μL per well. Luminescence measurement was conducted over a 5 s period, 2 s after reagent was automatically injected with Firefly luciferase reagent.

Statistical analysis

Antiandrogen receptor activity and PSA concentration were statistically evaluated using one-way ANOVA followed by Holm-Sidak's multiple comparisons test. $p < 0.05$ was considered statistically significant. All statistical analyses was performed in GraphPad Prism 7.01.

Results

Cell viability

To ensure that the studies on antiandrogenic activity were performed at non-cytotoxic concentrations, the cell viability of AR-EcoScreen and LNCaP cells were investigated after exposure to magnolol and analogs. We found that 1, 2 and 9 were cytotoxic at 33 and 100 μM and that 6 and 7 were cytotoxic at 100 μM . All other compounds and concentrations were non-cytotoxic (Fig. 2).

In LNCaP cells 1 was cytotoxic in both 33 and 100 μM and 6 was cytotoxic at 100 μM , while all other studied analogs (4, 7 and 11) were non-cytotoxic in the concentrations used (Fig. 3).

Antiandrogen receptor activity

The antiandrogenic receptor activity was studied in AR-EcoScreen cells, activated by DHT pretreatment. Antiandrogenic activity was exerted by all test compounds, except 4 and 10 (Fig 4). IC_{50} values (concentration causing 50% inhibition of the DHT-induced activity) varied between 7 and 86 μM . The IC_{50} values increased in the order $1 > 2 > 7 > 6 > 3 > 11 > 8 > 5$. All cytotoxic concentrations were excluded from analysis. As antiandrogenic activities can result from cytotoxicity it is worth noting that all analogs, except 4 and 10, had statistically significant antiandrogenic activities at non-cytotoxic concentrations. Compound 9 was cytotoxic at 33 and 100 μM , but showed statistically significant antiandrogenic activities also at lower, non-cytotoxic concentrations. However, the inhibitory effect in the non-cytotoxic concentrations did not reach 50% effect, and an IC_{50} value could therefore not be calculated for this compound.

Androgen receptor binding

The direct interaction of five of the test compounds with the ligand binding domain of AR was investigated with an assay, where the displacement of a synthetic ligand is measured by fluorescence polarization. DHT was used as a positive control. We found specific binding of 6 and to a lower extent also of 1 and 7 (Fig. 5).

Effects on PSA production

PSA production was measured in LNCaP cells as a biomarker of downstream androgenic signaling. Statistically significant decrease in PSA production was found after treatment of cells with 6, 7 at both 33 and 100 μM , and with 4 and 11 at 100 μM (Fig. 6). It should, however, be noted that compounds 6 and 7 were borderline cytotoxic (see Section 3.1) at 100 μM , which could cause a decrease in PSA production also without an effect on the AR signaling. The decrease in PSA production following exposure to 100 μM of 6 should therefore be interpreted with caution. The decrease of PSA production with 1 occurred at cytotoxic concentrations, while the other compounds and concentrations were not cytotoxic in LNCaP cells (Fig. 3).

Antiandrogenic activity after passage through a layer of Caco-2 cells

All the tested compounds, including the prostate cancer medical drugs abiraterone and enzalutamide, and the positive antiandrogenic reference substance OHF, drastically decreased the DHT-induced androgenic activity in AR-EcoScreen cells (Fig. 7). Analog 6, 7 and

AR EcoScreen cells

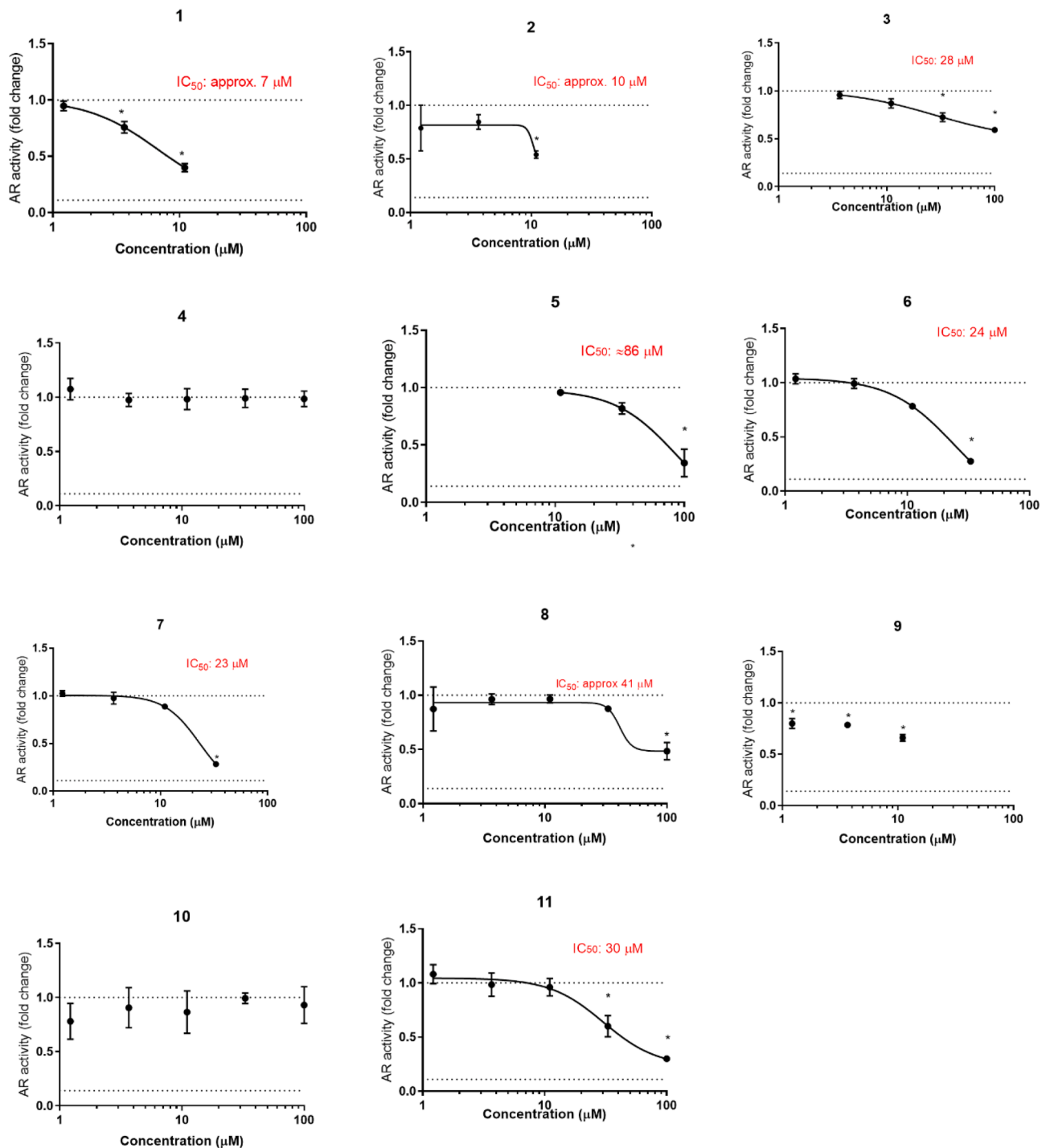


Fig. 4. Antiandrogen receptor activity of magnolol (1) and the ten analogs in AR-EcoScreen cells pretreated with DHT to activate AR signaling. For identification of analogs, see Fig. 1. Fold change compared to control expressed as mean \pm SD; $n = 4$. * $p < 0.05$ compared to vehicle control.

11 showed a dose-response in antiandrogenic activities, which were found to be statistically significantly different from the DMSO vehicle DHT treated control (DMSO/VC/DHT).

Trans epithelial electrical resistance

Trans epithelial electrical resistance (TEER) was measured across the Caco-2 monolayers at the start and the end of the experiments, as an

indicator of the integrity of the monolayers. Compared to the DHT control, TEER was statistically significantly decreased at all tested concentrations of 6 and 7 and at 100 μM of 11 and at 10 μM of OHF, abiraterone and enzalutamide, at most time points (Fig. 8). Effects on TEER were present already after 10 min incubation with test compounds and remained at a similar level at longer incubation times.

AR Polar Screen

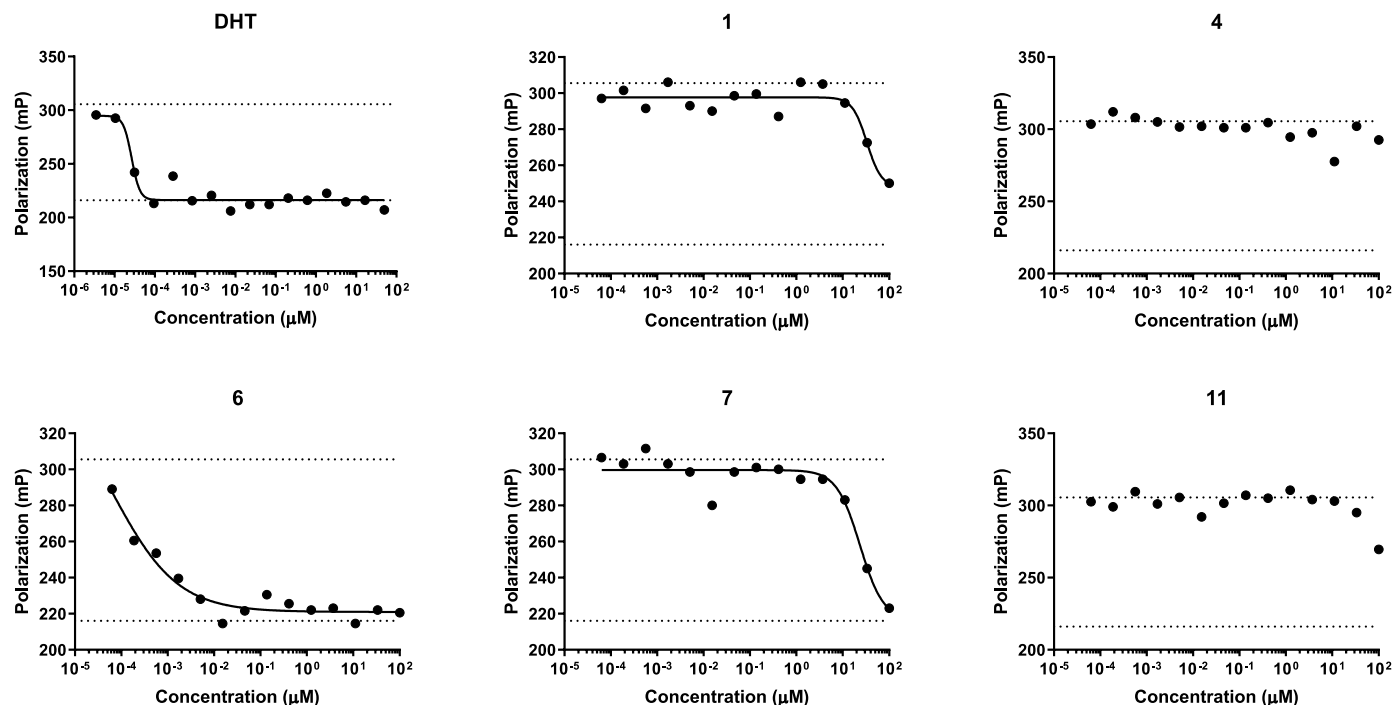


Fig. 5. Androgen receptor binding of DHT as a positive control, magnolol (1) and four analogs assayed with AR Polar Screen. A decrease in polarization indicates that the compound can bind to the ligand binding domain of AR. For identification of analogs, see Fig. 1. Mean; $n = 2$.

PSA production, LNCaP cells

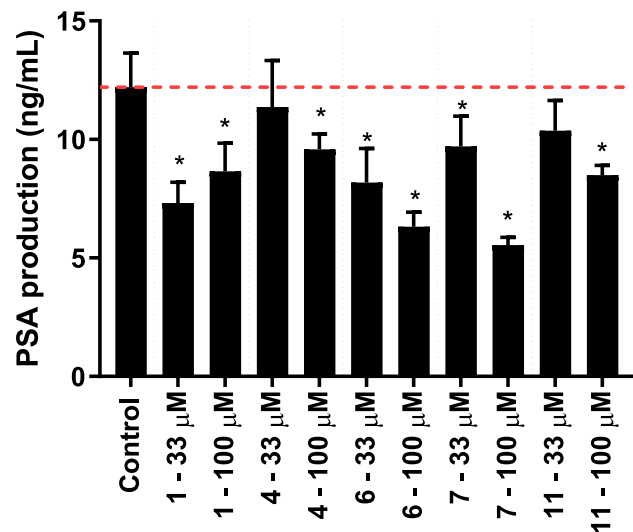


Fig. 6. Concentration of prostate-specific antigen (PSA) in a culture of LNCaP cells following 24 h exposure to magnolol (1) or four analogs. For identification of analogs, see Fig. 1. PSA concentration was measured by ELISA. Mean \pm SD, $n = 3$ for exposed groups and 12 for vehicle control. * $p < 0.05$ compared to vehicle control.

Discussion

Prostate cancer is the most common cancer form in men and prevention and treatment is of utmost importance for public health. Plant-derived bioactive molecules, especially phenolic compounds, have

shown promising potential for chemoprevention and therapy of prostate cancer (Hao et al., 2022; Salehi et al., 2019). Magnolol has been demonstrated *in vitro* to affect various molecular targets involved in initiation and growth of prostate cancer cells (see Introduction). Prostate cancer cells are dependent on androgens for growth and progression. To our knowledge, this is the first study to show antiandrogenic activities of magnolol and magnolol analogs. The effects were partly mediated by direct interaction with the ligand binding domain of the androgen receptor. Downstream effects on androgenic signaling were demonstrated by inhibition of PSA production in prostate cancer cells. As an additional promising potential for clinical application, we could also demonstrate antiandrogenic activity of magnolol analogs after passage through human intestinal Caco-2 cells.

The studied biological effects varied between the 11 tested magnolol compounds. Cytotoxicity was determined in AR-EcoScreen cells, where magnolol (1) and compound 2 were the most cytotoxic ones. Both compounds are unsubstituted in the bisphenyl rings apart from the OH-groups at position 6 and 6' and unsubstituted allyl- or alkyl-chain at the 3 and 3' positions. Compound 1 but not compound 3 was cytotoxic at the higher concentrations, indicating that methylation of the OH-groups in positions 6 and 6' in the bisphenyl ring protects from cytotoxicity. Similarly, compound 2, but not 4 and 5, was cytotoxic, indicating that hydroxylation and acetoxylation of the alkyl chains protects from cytotoxicity.

Similar results on cytotoxicity were observed in the prostate cancer LNCaP cells. Magnolol (1) had a high cytotoxicity, while no cytotoxicity was detected after treatment of cells with compounds 4 and 11, and a small decrease in cell viability was detected with compounds 6 and 7.

Cytotoxicity can have different implications *in vitro* studies on anti-cancer effects. Antiproliferative effects specifically towards cancer cells may suggest a clinical potential, while general cytotoxicity may indicate a risk of systemic toxicity and less clinical value. In the treatment of castrate-resistant prostate cancer, antiandrogenicity is the desired

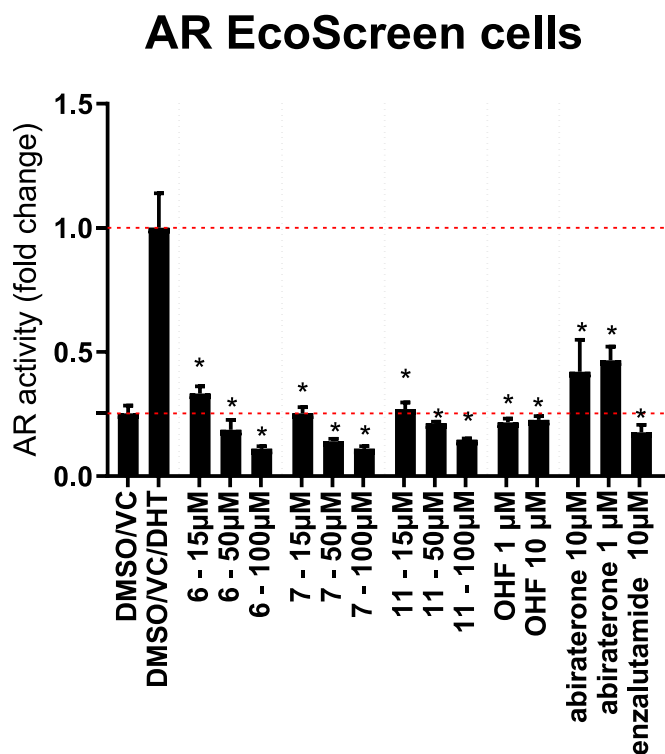


Fig. 7. Androgen receptor activity in AR-EcoScreen cultured in the lower compartment of a transwell plate with a tight layer of Caco-2 cells on the semipermeable insert. Magnolol analogs or clinically used prostate cancer drugs were applied in the upper compartment of the transwell plates at the indicated concentrations, together with DHT to activate AR signaling, and incubated for 4 h. Following 4 h incubation, the insert was removed and the AR-EcoScreen cells were left to incubate for an additional 20 h. For identification of analogs, see Fig. 1. Mean \pm SD; $n = 3$. * $p < 0.05$ compared to DMSO/VC/DHT treated control.

biological effect. A specific antiandrogenic effect, in the absence of cytotoxicity would be the most favorable. In *in vitro* assays for antiandrogen receptor activity, as used in the present study, androgen receptor activity is induced by DHT and the capacity of test substances to reduce the androgen activity is measured. A lower number of viable cells, due to cytotoxicity of the test substance, would give a false impression of antiandrogenicity. Consequently, antiandrogenic activity can only be accurately determined at non-cytotoxic concentrations of the test compound.

All compounds, except **4** and **10**, exhibited antiandrogenic activity at non-cytotoxic concentrations. Highest antiandrogenic activity was observed after treatment of cells with **1**, **2**, **7**, **6** and **3** (IC_{50} from 7 to 28 μ M), of which all compounds had unsubstituted allyl- or alkyl-chains at the 3 and 3' positions. Antiandrogenic activity, although lower, were observed after treatment with compounds **11**, **8** and **5** (IC_{50} 30 to 86 μ M), of which compounds **11** and **5** were acetoxyated on the alkyl-chain at the 3 and 3' positions. The compounds with no antiandrogenic activity, **4** and **10**, were the only compounds with OH-groups on the alkyl-chain at the 3 and 3' positions. Of the compounds with antiandrogenic activity, compounds **6**, **7** and **1** interacted directly with the ligand binding domain of the androgen receptor, while **4** and **11**, which had hydroxylated or acetoxyated alkyl chains at the 3 and 3' positions, did not.

Measurement of prostate-specific antigen (PSA) was first introduced in clinic to verify the response to treatment of prostate cancer and later used as a screening test to detect prostate cancer (Salehi et al., 2019). In the present study we tested PSA production in LNCaP cells after treatment with magnolol and four selected analogs. Compounds **6** and **7** were selected due to antiandrogenic activities and androgen receptor ligand

binding properties. Both compounds decreased PSA production at non-cytotoxic concentrations, demonstrating downstream effects on androgenic signaling. Compound **11**, which was selected as a compound with antiandrogenic activity and without ligand-binding capacity, reduced the PSA production at the higher concentration. Compound **4**, which neither had antiandrogenic activity nor ligand-binding capacity, caused a minor but statistically significant reduction in PSA production. The effect could either be spurious or due to effects on other enzymes in the steroidogenesis (e.g. an inhibitory effect on 17β -hydroxysteroid dehydrogenase or a stimulation of the activity of CYP19/aromatase), or caused by an unspecific non-androgenic mechanism.

Besides effects on the molecular targets, bioavailability and pharmacokinetic stability is essential for clinical application of drug candidates. Similar to other dietary polyphenols, such as resveratrol and catechins, magnolol has a low bioavailability and is rapidly conjugated with glucuronic or sulfuric acid (Sarrica et al., 2018). To improve the pharmacokinetic characteristics and the potential for clinical use, chemical analogs have been synthesized and tested in the present study. To investigate bioavailability of the test compounds we established an experimental model for determination of antiandrogenic activity after gastrointestinal absorption, by using human Caco-2 cells in the upper compartment and AR-EcoScreen cells in the lower compartment of transwells. The three magnolol analogs, which caused a reduction of PSA production (**6**, **7** and **11**) were tested together with the positive reference compound OHF and two prostate cancer drugs, abiraterone and enzalutamide. Antiandrogenic effects were observed after passage through Caco-2 cells by all compounds and for the three antiandrogenic magnolol analogs the effects were dose-related. These results are promising for the development of magnolol analogues as anti-prostate cancer drugs, as it indicates the potential for a good bioavailability after oral administration. It should, however, be noted that the transepithelial electrical resistance of the Caco-2 cell monolayer (a measurement of the integrity of the cell monolayer) was statically decreased by all studied compounds, at least in the highest concentration. Further studies are needed to investigate if the transport of the test compounds over the Caco-2 cell layer is via passive diffusion or active transport through the cells, or via paracellular transport between the cells following a loosening of the tight junctions in the cell layer. Interestingly, also the already clinically used drugs enzalutamide and abiraterone show the same effect of decreasing the transepithelial electrical resistance of the Caco-2 cell monolayer.

A similar approach to test synthetic analogs with presumably better pharmacokinetic properties was applied for another plant-derived phenol, resveratrol, with documented effects on androgen synthesis (Oskarsson et al., 2014) and inhibition of androgen receptor activity (Lundqvist et al., 2017). The most potent analogs were acetylated and methylated analogs, which also had longer half-lives, increased AUC and volume of distribution, compared to resveratrol (Liang et al., 2013).

In this study, we have shown that magnolol and multiple magnolol analogs exert antiandrogenic effects at non-cytotoxic concentrations, that this antiandrogenic effect for some of these compounds is mediated via a direct interaction with the AR, and for selected compounds that they have the capacity to decrease the production of PSA (a downstream biomarker of androgenic signaling). Further, we have shown that selected analogs are able to pass over a tight layer of Caco-2 cells, an experimental model to evaluate gastrointestinal absorption of compounds, which indicates a potential for good bioavailability of these compounds after oral administration. In conclusion, magnolol analogs constitute an interesting group of compounds worthy of further evaluation as potential anti-prostate cancer therapeutics due to their antiandrogenic properties, the potential for good bioavailability after oral administration and potentially preferential properties from a pharmacokinetic perspective.

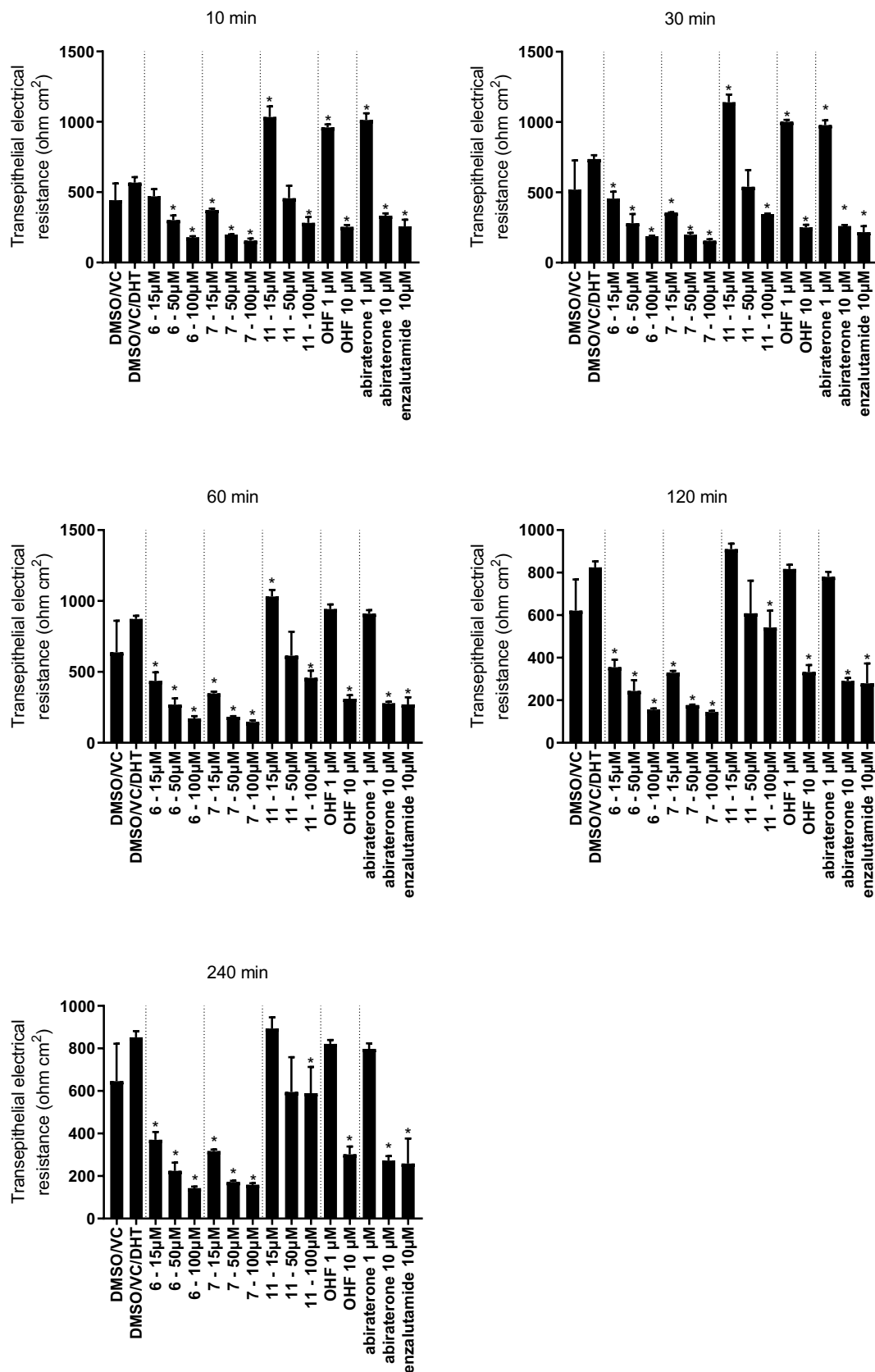


Fig. 8. Transepithelial electrical resistance (TEER) of the Caco-2 cell layer following 10, 30, 60, 120 and 240 min of exposure to controls, magnolol, analogs and clinically used prostate cancer drugs. For identification of analogs, see Fig. 1.

Author agreement

I hereby certify that all authors have seen and approved the final version of this manuscript. Further, I certify that the article is our original work, that is has not been published before and that it is not under consideration for publication elsewhere.

CRediT authorship contribution statement

Agneta Oskarsson: Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft. **Geeta Mandava:** Investigation, Methodology, Validation, Writing – review & editing. **Corrado Tringali:** Conceptualization, Investigation, Methodology, Validation, Supervision, Writing – review & editing. **Luana Pulvirenti:** Investigation, Methodology, Validation, Writing – review & editing. **Vera Muccilli:** Investigation, Methodology, Validation, Writing – review & editing. **Johan Lundqvist:** Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

- Barnard, M., Mostaghel, EA., Auchus, RJ., Storbeck, K.-H., 2020. The role of adrenal derived androgens in castration resistant prostate cancer. *J. Steroid Biochem. Mol. Biol.* 197, 105506 <https://doi.org/10.1016/j.jsbmb.2019.105506>.
- Baschieri, A., Pulvirenti, L., Muccilli, V., Amorati, R., Tringali, C., 2017. Chain-breaking antioxidant activity of hydroxylated and methoxylated magnolol derivatives: the role of H-bonds. *Org. Biomol. Chem.* 15 (29), 6177–6184. <https://doi.org/10.1039/c7ob01195d>.
- Chandrasekar, T., Yang, J.C., Gao, A.C., Evans, C.P., 2015. Mechanisms of resistance in castration-resistant prostate cancer (CRPC). *Transl. Androl. Urol.* 4 (3), 365–380. <https://doi.org/10.3978/j.issn.2223-4683.2015.05.02>.
- Chen, Ya-H, Lu, M.-H., Guo, D.-S., Zhai, Y.-Y., Miao, D., Yue, J.-Y., Yuan, C.-H., Zhao, M.-M., An, De-R, 2019. Antifungal effect of magnolol and honokiol from magnolia officinalis on alternaria alternata causing tobacco brown spot. *Molecules* 24 (11), 2140. <https://doi.org/10.3390/molecules24112140>.
- Fuchs, A., Baur, R., Schoeder, C., Sigel, E., Müller, CE., 2014. Structural analogues of the natural products magnolol and honokiol as potent allosteric potentiators of GABAA receptors. *Bioorg. Med. Chem.* 22 (24), 6908–6917. <https://doi.org/10.1016/j.bmc.2014.10.027>.
- GLOBOCAN, 2020. Global Cancer Statistics. Switzerland.
- Hao, Q., Wu, Y., Vadgama, JV., Wang, P., 2022. Phytochemicals in inhibition of prostate cancer: evidence from molecular mechanisms studies. *Biomolecules* 12 (9), 1306. <https://doi.org/10.3390/biom12091306>.
- Huang, Yeqing, Xiangyu, Z., Xiaowen, Z., Feng, W., Weidong, Z., Guangyuan, Z., Jun, X., Ming, C., 2017. Magnolin inhibits prostate cancer cell growth in vitro and in vivo. *Biomed. Pharmacother.* 87, 714–720. <https://doi.org/10.1016/j.biopha.2017.01.010>.
- Hwang, E.-S., Park, K.-K., 2010. Magnolol suppresses metastasis via inhibition of invasion, migration, and matrix metalloproteinase-2/-9 activities in PC-3 human prostate carcinoma cells. *Biosci. Biotechnol. Biochem.* 74 (5), 961–967. <https://doi.org/10.1271/bbb.90785>.
- Jada, S., Doma, M.R., Singh, P.P., Kumar, S., Malik, F., Sharma, A., Khan, I.A., Qazi, G.N., Sampath Kumar, H.M., 2012. Design and synthesis of novel magnolol derivatives as potential antimicrobial and antiproliferative compounds. *Eur. J. Med. Chem.* 51, 35–41. <https://doi.org/10.1016/j.ejmech.2011.12.039>.
- Lee, B., Kwak, J.-H., Huang, S.-W., Jang, J.-Y., Lim, S., Kwak, Y.-S., Lee, K., Kim, H.S., Han, S.-B., Hong, J.-T., Lee, H., Song, S., Seo, S.-Y., Jung, J.-K., 2012. Design and synthesis of 4-O-methylhonokiol analogs as inhibitors of cyclooxygenase-2 (COX-2) and PGF₂ production. *Bioorg. Med. Chem.* 20 (9), 2860–2868. <https://doi.org/10.1016/j.bmc.2012.03.028>.
- Lee, D.-H., Szczepanski, M.-J., Lee, Y.J., 2009. Magnolol induces apoptosis via inhibiting the EGFR/PI3K/Akt signaling pathway in human prostate cancer cells. *J. Cell. Biochem.* 106 (6), 1113–1122. <https://doi.org/10.1002/jcb.22098>.
- Liang, Li, Liu, X., Wang, Q., Cheng, S., Zhang, S., Zhang, M., 2013. Pharmacokinetics, tissue distribution and excretion study of resveratrol and its prodrug 3,5,4'-Tri-O-acetylresveratrol in rats. *Phytomedicine* 20 (6), 558–563. <https://doi.org/10.1016/j.phymed.2012.12.012>.
- Lin, C.-F., Hung, C.-F., Aljuffali, IA., Huang, Yu-L, Liao, W.-C., Fang, J.-Y., 2016. Methylation and esterification of magnolol for ameliorating cutaneous targeting and therapeutic index by topical application. *Pharm. Res.* 33 (9), 2152–2167. <https://doi.org/10.1007/s11095-016-1953-x>.
- Lin, J.M., Prakasha Gowda, A.S., Sharma, AK., Amin, S., 2012. In vitro growth inhibition of human cancer cells by novel honokiol analogs. *Bioorg. Med. Chem.* 20 (10), 3202–3211. <https://doi.org/10.1016/j.bmc.2012.03.062>.
- Lin, Yiping, Yuke, Li, Yuanlian, Z., Bin, T., Xiaolan, Qu, Qianghua, Y., Ying, S., 2021. Pharmacology, toxicity, bioavailability, and formulation of magnolol: an update. *Front. Pharmacol.* 12.
- Litwin, MS., Tan, H.-J., 2017. The diagnosis and treatment of prostate cancer: a review. *JAMA* 317 (24), 2532–2542. <https://doi.org/10.1001/jama.2017.7248>.
- Lundqvist, J., Tringali, C., Oskarsson, A., 2017. Resveratrol, piceatannol and analogs inhibit activation of both wild-type and T877A mutant androgen receptor. *J. Steroid Biochem. Mol. Biol.* 174, 161–168. <https://doi.org/10.1016/j.jsbmb.2017.08.016>.
- McKeown, BT., Hurta, RA.R., 2015. Magnolol affects cellular proliferation, polyamine biosynthesis and catabolism-linked protein expression and associated cellular signaling pathways in human prostate cancer cells in vitro. *Funct. Foods Health Dis.* 5 (1), 17–33. <https://doi.org/10.31989/ffhd.v5i1.163>.
- Micco, Di, Simone, Pulvirenti, L., Bruno, I., Terracciano, S., Russo, A., Vaccaro, MC., Ruggiero, D., Muccilli, V., Cardullo, N., Tringali, C., Riccio, R., Bifulco, G., 2018. Identification by inverse virtual screening of magnolol-based scaffold as new tankyrase-2 inhibitors. *Bioorg. Med. Chem.* 26 (14), 3953–3957. <https://doi.org/10.1016/j.bmc.2018.06.019>.
- Mori, K., Mostafaei, H., Pradere, B., Motlagh, R.S., Quhal, F., Laukhina, E., Schuettfort, VM., Abufaraj, M., Karakiewicz, PL., Kimura, T., Egawa, S., Shariat, SF., 2020. Apalutamide, enzalutamide, and darolutamide for non-metastatic castration-resistant prostate cancer: a systematic review and network meta-analysis. *Int. J. Clin. Oncol.* 25 (11), 1892–1900. <https://doi.org/10.1007/s10147-020-01777-9>.
- Oskarsson, A., Spatafora, C., Tringali, C., Ohlsson Andersson, Å., 2014. Inhibition of CYP17A1 activity by resveratrol, piceatannol, and synthetic resveratrol analogs. *Prostate* 74 (8), 839–851. <https://doi.org/10.1002/pros.22801>.
- Posdzhich, P., Darr, C., Hilsner, T., Wahl, M., Herrmann, K., Hadaschik, B., Grünwald, V., 2023. Metastatic prostate cancer—a review of current treatment options and promising new approaches. *Cancers* 15 (2), 461. <https://doi.org/10.3390/cancers15020461>.
- Pulvirenti, L., Muccilli, V., Cardullo, N., Spatafora, C., Tringali, C., 2017. Chemoenzymatic synthesis and α -glucosidase inhibitory activity of dimeric neolignans inspired by magnolol. *J. Nat. Prod.* 80 (5), 1648–1657. <https://doi.org/10.1021/acs.jnatprod.7b00250>.
- Ranaware, A.M., Banik, K., Deshpande, V., Padmavathi, G., Roy, N.K., Sethi, G., Fan, Lu, Kumar, A.P., Kunnumakkara, AB., 2018. Magnolol: a neolignan from the magnolia family for the prevention and treatment of cancer. *Int. J. Mol. Sci.* 19 (8), 2362. <https://doi.org/10.3390/ijms19082362>.
- Salehi, B., Fokou, P.V.T., Yamthe, L.R.T., Tali, B.T., Adetunji, C.O., Rahavian, A., Mudau, F.N., Martorell, M., Setzer, WN., Rodrigues, CF., Martins, N., Cho, WC., Sharifi-Rad, J., 2019. Phytochemicals in prostate cancer: from bioactive molecules to upcoming therapeutic agents. *Nutrients* 11 (7), 1483. <https://doi.org/10.3390/nu11071483>.
- Sarrica, A., Kirika, N., Romeo, M., Salmona, M., Diomedea, L., 2018. Safety and toxicology of magnolol and honokiol. *Planta Med.* 84 (16), 1151–1164. <https://doi.org/10.1055/a-0642-1966>.
- Wang, X., Liu, Q., Fu, Y., Ding, R-Bo, Qi, X., Zhou, X., Sun, Z., Bao, J., 2022. Magnolol as a potential anticancer agent: a proposed mechanistic insight. *Molecules* 27 (19), 6441. <https://doi.org/10.3390/molecules27196441>.
- Zhang, J., Chen, Z., Huang, X., Shi, W., Zhang, R., Chen, M., Huang, H., Wu, L., 2019. Insights on the multifunctional activities of magnolol. *Biomed. Res. Int.* 2019, e1847130 <https://doi.org/10.1155/2019/1847130>.