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Antiandrogenic activity of anthranilic acid ester derivatives as novel lead structures to inhibit prostate cancer cell proliferation

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A plant extract from the fruits of saw palmetto, which is currently used to treat the androgen-dependent benign prostatic hyperplasia and PCa, served as source for new structure variants. We investigated the antiandrogenic potential of an ethanolic total extract and one of its main aromatic components anthranilic acid. An androgen receptor-antagonistic (antiandrogenic) effect of the extract was evident, and although anthranilic acid itself revealed no remarkable effect, its methyl ester, methyl anthranilate, exhibited antiandrogenic potential. Based on this chemical structure, we synthesized and investigated the antiandrogenic activity of four AnA ester derivatives, which were either novel or only little described in literature. These AnA esters inhibit the androgen-dependent transactivation of both the wild-type (wt) androgen receptor and the androgen receptor point mutant T877A, which often occurs in refractory PCa. Moreover, they inhibit the androgen receptor-induced expression of the endogenous prostate-specific antigen. Importantly, AnA esters repress the growth of human PCa cells. Deletion analyses of androgen receptor propose that the antiandrogenic effect of anthranilic acid esters is mediated by the ligand-binding domain, most likely through direct binding, without affecting androgen receptor protein levels. Taken together, the data suggest antiandrogenic potential of anthranilic acid ester derivatives, which can serve as lead structures for novel antiandrogens.

Key words: anthranilic acid, AR antagonism, ester derivative, methyl anthranilate, prostate cancer, *Sabal serrulata, Serenoa repens*

Abbreviations

AA, atraric acid; AnA, anthranilic acid; AR, androgen receptor; ARE, androgen response element; BPH, benign prostatic hyperplasia; CV1, kidney cell line from green monkey, lacking endogenously expressed functional AR, GR, ER, PR and TR; DBD, DNA-binding domain; DHT, dihydrotestosterone; FA, flufenamic acid; Fl, flutamide; GR, human glucocorticoid receptor; LBD, ligand-binding domain; LNCaP, lymph node carcinoma prostate cell line; luc, luciferase; MMTV, mouse mammary tumor virus; PCa, prostate cancer; PSA, prostate-specific antigen; PR-B, human progesterone receptor; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; R1881, methyltrienolone; RLU, relative luciferase units; *S. repens*, *Serenoa repens*.

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The risk of men to develop androgen-dependent diseases as benign prostatic hyperplasia (BPH) and prostate cancer (PCa) increases rapidly with rising age. Among men, PCa is, meanwhile, the second leading cause of cancer death in western countries (1). The androgen receptor (AR), which is the most important drug target for PCa hormone therapies, is activated by androgens, mainly by 5α-dihydrotestosterone (DHT). The AR is a member of the nuclear hormone receptor (NHR) superfamily, a large group of ligand-dependent transcription factors (2), and mainly structured into four functional domains: (I) the N-terminal domain with the major transactivation function, (II) a highly conserved DNA-binding domain, (III) a less conserved hinge domain, and (VI) a ligand-binding domain (LBD) at the C-terminus (3). Androgen binding results in an activated AR that subsequently translocates into the nucleus, homodimerizes, and binds to androgen-response elements (AREs) of several target genes (4). Androgens promote the growth of both normal prostate and of PCa. In case of PCa, hormone therapy using AR antiandrogens (AR antagonists) is an efficient manner to block AR-mediated transactivation and tumor growth. Interestingly, mutations of the AR such as the T877A mutant, which often occurs in refractory PCa (5), turn the active metabolite 2-hydroxyflutamide of the commonly used complete antiandrogen flutamide into a potent AR agonist, resulting in enhanced tumor growth (6). In general, it is important to identify and synthesize new antiandrogens with other characteristics than the currently used. Plant extracts from the fruits of saw palmetto are one possible source to search for new antiandrogen scaffolds.

Saw palmetto fruit extracts are successfully used since more than 100 years for the treatment of problems of the urogenital tract (7). The fruits originate from a scrubby palm *Serenoa repens* (W. Bartram) (Arecacea) found growing in sandy soil along costal areas in the southeast of the United States (8). Anti-inflammatory (9),

antiproliferative (10), and antiandrogenic effects (11) of saw palmetto fruit extracts have been shown. Many investigations have been made concerning the antiandrogenic effect of the extracts' main component, fatty acids (12), but so far antiandrogenic activity of aromatic ingredients has not yet been described.

Materials and Methods

The investigated plant extract was an ethanolic total extract from the fruits of *Serenoa repens* (96 %). It was supplied by Finzelberg GmbH & Co. KG, Andernach (batch: 03120256).

Dexamethasone, progesterone, dihydrotestosterone (DHT), methyl anthranilate (MA), anthranilic acid (AnA), and flufenamic acid (FA) were obtained from Sigma. Methyltrienolone (R1881) was obtained from Perkin Elmer and from LKT Laboratories, Inc. All test compounds were dissolved in ethanol and/or dimethyl sulfoxide (DMSO). These compounds were added to the culture medium such that the final concentration of ethanol and/or DMSO did not exceed 0.1%. Control incubations (no test compounds) were performed in the presence of only 0.1% ethanol and/or DMSO.

NMR spectra were recorded on a JEOL ECA-500 (500 MHz) spectrometer and referenced to the solvent resonances in $CDCl_3$. Chemical shifts are reported in ppm and are assigned as singlets (s), doublets (d), triplets (t), quartets (q), sextets, and multiplets (m). The abbreviation br stands for broad signal. EI-MS and HR-EI-MS spectra were obtained with a Micromass VG 7070H and a Micromass AutoSpec mass spectrometer employing an ionizing energy of 70 eV. Infrared spectra of the plain substances were measured with a Bruker Alpha-P IR spectrometer. The refraction indexes were obtained on a Zeiss refractometer type 92. The syntheses were carried out with a previously described modified procedure (13).

Butan-2-yl-2-aminobenzoate (E1)

To a solution of isatoic anhydride (4.08 g, 25 mmol) in racemic 2-butanol (40 mL), sodium hydroxide (0.05 g, 1.25 mmol) was added. The reaction mixture was stirred and heated until moderate evolution of CO_2 occurred (90 °C). This temperature was maintained till gas evolution had ceased. The mixture was cooled and diluted with water (120 mL). The product settled out as immiscible oil, which was separated and purified by vacuum distillation. The product, a colorless oil (2.53 g, 52%), passed over at 126 °C and 5 Torr. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.97 (t, 3H), δ 1.32 (d, 3H), δ 1.79-1.61 (m, 2H), δ 5.06 (sextet, 1H), δ 5.72 (br s, 2H), δ 6.64 (t, 2H), δ 7.25 (t, 1H), δ 7.87 (d, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm) 9.7, 19.6, 28.9, 72.1, 111.5, 116.1, 116.6, 131.2, 133.8, 150.4, 167.8; EI-MS (70 eV): m/z (rel.int.) = 193 $[M]^+$ (100), 137 (87), 119 (86), 92 (29); HR-EI-MS: m/z = 193.1108; $C_{11}H_{15}NO_2$ $[M]^+$ requires 238.1226; IR (plain): λ_{max} = 3480, 3370, 2970, 1680, 1240, 1090; n_D^{20} = 1.5411.

Butan-2-yl-2-methylaminobenzoate (E2)

To a solution of N-methylisatoic anhydride (5.0 g, 28 mmol) in racemic 2-butanol (40 mL), sodium hydroxide (0.1 g, 2.5 mmol) was added. The reaction mixture was stirred and heated until moderate evolution of CO_2 occurred (85 °C). This temperature was maintained till gas evolution had ceased. The mixture was cooled and diluted with water (120 mL). The product settled out as immiscible oil, which was separated and purified by vacuum distillation. The product, a yellow oil (2.92 g, 50 %), passed over at 110 °C and 0.35 Torr. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.96 (t, 3H), δ 1.31 (d, 3H), δ 1.78-1.59 (m, 2H), δ 2.90 (d, 3H), δ 5.03 (sextet, 1H), δ 6.58 (t, 1H), δ 6.66 (d, 1H), δ 7.37 (t, 1H), δ 7.69 (br s, 1H), δ 7.92 (d, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm) 9.8, 19.6, 29.0, 29.5, 71.9, 110.66, 110.72, 114.2, 131.5, 134.4, 152.0, 168.4; EI-MS (70 eV): m/z (rel.int.) = 207 $[M]^+$ (100), 151 (97), 134 (68), 132 (55), 105 (78), 77 (30); HR-EI-MS: m/z = 207.1260; $C_{12}H_{17}NO_2$ $[M]^+$ requires 207.1259; IR (plain): λ_{max} = 3370, 2980, 1670, 1580, 1430, 1230; n_D^{20} = 1.5442.

Prop-2-enyl-2-methylaminobenzoate (E3)

To a solution of N-methylisatoic anhydride (5.0 g, 28 mmol) in allyl alcohol (40 mL), sodium hydroxide (0.1 g, 2.5 mmol) was added. The reaction mixture was stirred and heated until moderate evolution of CO_2 occurred (85 °C). This temperature was maintained till gas evolution had ceased. The mixture was cooled and diluted with water (120 mL). The product settled out as immiscible oil, which was separated and purified by vacuum distillation. The product, a yellowish oil (3.40 g, 64%), passed over at 98-99 °C and 0.22 Torr. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.91 (d, 3H), δ 4.77 (d, 2H), δ 5.27 (d, 1H), δ 5.40 (d, 1H), δ 6.03 (m, 1H), δ 6.59 (t, 1H), δ 6.67 (d, 1H), δ 7.39 (t, 1H), δ 7.64 (br s, 1H), δ 7.95 (d, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm) 29.5, 64.7, 109.7, 110.9, 114.3, 117.7, 131.5, 132.6, 134.7, 152.1, 168.2; EI-MS (70eV): m/z (rel.int.) = 191 $[M]^+$ (100), 150 (56), 132 (80), 116 (29), 105 (73), 91 (30), 77 (43); HR-EI-MS: m/z = 191.0946; $C_{11}H_{13}NO_2$ $[M]^+$ requires 191.0946; IR (plain): λ_{max} = 3380, 2900, 1680, 1580, 1430, 1230; n_D^{20} = 1.5699.

1-Methyoxypropan-2-yl-2-methylaminobenzoate (E4)

To a solution of N-methylisatoic anhydride (5.0 g, 28 mmol) and 1-methoxy-2-propanol (2.52 g, 28 mmol) in 1,4-dioxane (40 mL), sodium hydroxide (0.1 g, 2.5 mmol) was added. The reaction mixture was stirred and heated until moderate evolution of CO_2 occurred (90 °C). This temperature was maintained till gas evolution had ceased. The mixture was cooled and diluted with water (120 mL). The product settled out as immiscible oil which was separated. Purification was carried out by vacuum distillation to give a yellow oil (2.9 g, 46 %) passed over at 117–118 °C and 0.25 Torr. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.33 (d, 3H), δ 2.90 (d, 3H), δ 3.39 (s, 3H), δ 3.53 (m, 2H), δ 5.27 (m, 1H), δ 6.58 (t, 1H), δ 6.65 (d, 1H), δ 7.37 (t, 1H), δ 7.64 (br s, 1H), δ 7.92 (d, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm) 16.8, 29.5, 59.2, 69.0, 75.2, 110.2, 110.6 114.2, 131.6 134.5, 152.0, 168.0; EI-MS (70 eV): m/z (rel.int.) = 223 $[M]^+$ (100), 165 (25), 151 (79), 134 (66), 132 (45), 105 (62), 73 (32); HR-EI-MS: m/z = 223.1199; $C_{12}H_{17}NO_3$ $[M]^+$ requires 223.1208; IR (plain): λ_{max} = 3380, 2930, 1670, 1580, 1520, 1230; n_D^{20} = 1.5538.

Plasmids

The plasmid pMMTV-luc, which contains a luciferase reporter gene driven by the mouse mammary tumor virus long terminal repeats

responsive to androgens, was described previously (11,14). The expression vectors for the human AR or AR-T877A, pSG5-hAR or pSG5-hAR T877A were described previously (15). The plasmid for the expression of human GR is described in reference (16). Human PR-B expression vector was kindly provided by P. Chambon (Strasbourg, France).

Reporter assays

Reporter assays were described elsewhere (13).

Cell growth assays

Human prostate carcinoma lymph node carcinoma prostate cell line (LNCaP) cells (17) were cultured in RPMI-1640 medium (Invitrogen), supplemented with 10% (v/v) fetal calf serum (FCS) (Invitrogen), 1% (v/v) penicillin and streptomycin (Invitrogen), 1% (v/v), and 1% (v/v) sodium pyruvate (Sigma). For cell growth assays, cells were seeded onto 24-well tissue culture plates (Greiner) at 5×10^3 cells per well in appropriate medium containing 5% FCS in triplicates. After 48 h, cells were fed with fresh medium and treated with ethanol/DMSO or with the indicated compounds. Every second day, the media were replaced with fresh media together with freshly added compounds. The cells were trypsinized and counted using a counting cell chamber (Double Neubauer, Brand, Germany) at the indicated times.

Protein detection

A total of 25×10^3 LNCaP cells were seeded on 10-cm cell culture dishes into RPMI medium containing 5% FCS. After 24 h, cells were incubated with the indicated compounds at 37°C for additionally 3 days. Proteins were extracted by scrapping cells in ice cold PBS and pelleting cells by centrifugation at 1000 *g* for 5 min at 4°C. The cell pellet was resuspended in 500 μL NP-40 lysis buffer (0.1% SDS, 1% NP-40, 50 mM Tris, pH 8, 150 mM NaCl, 5 mM ETDA, 1 mM PMSF). The lysis was followed by three cycles of freezing (with liquid N_2) and thawing (37°C). The cell debris were removed by centrifugation at $12\,000 \times g$ for 15 min at 4°C. Equal amounts of protein (10 μg) were separated on SDS-PAGE and blotted onto a PVDF membrane (Millipore, Billerica, NA, USA). Western blot analysis was performed by using a mouse anti-AR antibody [F39.4.1 (BioGenex Laboratories, Fremont, CA, USA)] and the enhanced chemoluminescence detection method (Amersham Pharmacia Biotech). As secondary antibody, an anti-mouse IgG HRP-linked antibody was used (Santa Cruz, Santa Cruz, CA, USA).

Real-time RT-PCR

The real-time RT-PCR (qRT-PCR) was performed essentially as described previously (18) with specific primers for the detection of prostate-specific antigen (PSA) mRNA and β-actin mRNA for normalization. As modifications, 1.28×10^6 cells were seeded out directly in RPMI medium containing 10% charcoal-stripped FCS. After 2 days, cells were treated with the indicated compounds or solvent (ethanol) and, if indicated, with androgen (R1881) (final concentration of 1 nM) for 36 h.

Whole cell binding assay

The competitive whole cell binding assay was described elsewhere (18) with the modification that Cos-7 cells were transfected with the pSG5-hARwt plasmid and for normalization control together with the pCMV-lacZ expression vector.

Results

Antiandrogenic effect of an ethanolic total extract from saw palmetto fruits

We first investigated the antiandrogenic activity of an ethanolic total extract from saw palmetto fruits. Reporter gene assays were used to measure the antiandrogenic effect. For that purpose, CV1 cells that do not express endogenous steroid receptors such as AR, progesterone receptor (PR), glucocorticoid receptor (GR), and estrogen receptor, which may interfere with the assay system, were transiently cotransfected with the human AR-expression plasmid, a luciferase reporter gene with an androgen-sensitive MMTV promoter (19), and a constitutive active lacZ gene (pCMV-lacZ) for internal normalization. The slight repression of the AR transactivation in the absence of androgens is probably due to the inhibitory effect of *S. repens* on the 5α-reductase that has been shown in former studies (20). Therefore, cells were treated with a stable synthetic androgen (R1881) to avoid side-effects of metabolites of DHT together with the plant extract. Cotreatment of the cells with androgen and the *S. repens* extract resulted in about 50 % inhibition of the AR transactivation (Figure 1), which suggests an antiandrogenic activity.

Methyl anthranilate (MA) inhibits the wt AR and the T877A mutant without affecting the AR protein level

Investigations into compositions of saw palmetto fruits led to the identification of AnA (21), which we could also detect in the ethanolic total extract. Because natural compounds can exhibit stronger effects when the carboxylic acid group is esterified (22), we tested additionally the methyl ester of AnA, methyl anthranilate (MA). Interestingly, although AnA shows no remarkable inhibition of the AR-mediated transactivation, MA has inhibitory effects (Figure 2A). Performed dose curve experiments with the natural androgen DHT (Figure 2B) or the synthetic androgen R1881 (Figure 2C) confirmed the effect of MA and revealed a dose-dependent inhibition pattern. MA reduced androgen-induced AR-mediated transactivation at a concentration of 3×10^{-4} M significantly and slightly at a concentration of 10^{-4} M.

Effects of MA on the AR mutant T877A

The AR-T877A is an AR point mutant likely occurring in hormone therapy–resistant PCa (5). The exchange of one amino acid in the AR-LBD results in a loss of effectiveness of the currently used antiandrogen flutamide (Fl) and its metabolite hydroxyflutamide (OH-Fl). More broadly, Fl acts as agonist for AR-T877A and therefore increases tumor growth (6). In consideration of this drawback of antagonist treatment, new antiandrogens should exhibit advantages and not losing their intended effect. Therefore, the antiandrogenic

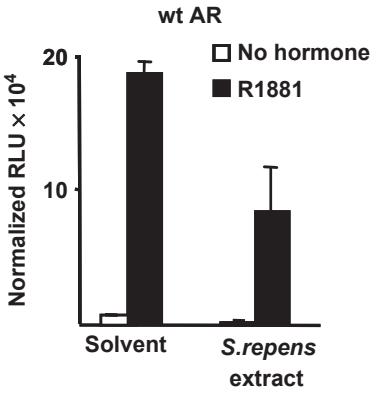


Figure 1: Antiandrogenic activity of an ethanolic total extract from saw palmetto fruits (*S. repens*). The extract was tested for its potential to inhibit the hormone-activated human wt androgen receptor (AR) in a reporter gene–based system. CV1 cells lacking functional endogenous steroid hormone receptors were transiently transfected with the expression vector for human AR and the androgen responsive reporter MMTV luciferase and subsequently treated with the plant extract (300 $\mu g/\mu L$). It was tested in the absence (white bars) and presence (black bars) of the synthetic androgen R1881 (30 pM). Solvent (ethanol) was used as negative control. The obtained luciferase values were normalized to the co-transfected internal control pCMV-lacZ, indicated as normalized relative light units (RLU). Error bars indicate the deviation of the mean of two wells. The experiments have been repeated at least three times.

activity of MA on this AR mutant was analyzed. The data suggest that MA still maintains its antiandrogenic potency for this mutant (Figure 2D) by detection of a significant decrease in the AR-T877A-mediated transactivation.

To investigate the effect of MA on the mRNA levels of the endogenous AR target gene PSA, quantitative real-time PCR (qRT-PCR) experiments were performed. The human androgen-dependent LNCaP cells that endogenously express the AR-T877A mutant were used as test system. The treatment of cells with MA resulted in an inhibition of the androgen-activated PSA expression (Figure 3). This suggests further that MA acts as an antiandrogen.

To test the possibility that the antiandrogenic effect of MA is achieved by degradation of the AR protein level. Western blot analysis with LNCaP cell extracts was conducted. AR protein degradation has been shown to be the subjacent mechanism of the known antiandrogen flufenamic acid (FA) (23). Therefore, FA treatment of LNCaP cells served as positive control. The AR protein levels were detected with an AR-specific antibody, and as loading control, β-actin antibody was used. In contrast to FA, MA treatment did not change the AR protein levels (Figure 4A). This indicates that MA treatment of PCa cells does not induce AR degradation. To analyze whether MA inhibits the AR through the LBD, an AR mutant lacking the LBD (ARΔLBD) was employed in reporter gene assays. Interestingly, the antiandrogen FA reduces the transactivation of ARΔLBD, suggesting that FA acts independent of the AR-LBD. As opposed to FA, the LBD deletion abrogates MA-mediated AR inhibition (Figure 4B), suggesting that the AR-LBD is required for MA-mediated inhibition of AR.

In summary, the data suggest that the AnA methyl ester MA inhibits the transactivation of both the wt AR and the AR-T877A mutant. Furthermore, MA reduces the PSA mRNA levels and requires the AR-LBD to exhibit an antiandrogenic effect.

Syntheses of AnA ester derivatives lead to more active antiandrogens

To study the influences of the ester alcohol of MA for antiandrogenic effect, we synthesized a derivative with an isobutyl group (E1) as a branched ester residue. We also combined this ester alcohol with a single methylated amino group (compare E1 and E2) as shown in Table 1. In addition, we synthesized ester alcohol derivatives with an unsaturated residue (E3) and an oxygen containing branched residue (E4), each combined with a single methylated amino group. The esters were produced by the reaction of isatoic anhydride or N-methyl isatoic anhydride and the pursuant alcohol with small quantities of NaOH as catalyst (24). E2-E4 are novel molecules and not listed in chemical databases.

Interestingly, all modifications increase the antiandrogenic activity compared to MA, at least tenfold (Figure 5). Dose curve experiments suggest that E2 and E4 are potent at a concentration of 3×10^{-5} M and E1 and E3 at a concentration of 10^{-5} M (Supporting Information). The calculated IC_{50} values of the synthesized compounds range from 6 μM (E3) to 24 μM (E4) compared with an IC_{50} of MA of 180 μM. The IC_{50} of E1 and E4 is about 18 μM. Comparing the IC_{50} of E2 with those of currently used AR antagonists for PCa therapy, the IC_{50} of E2 is only slightly superior (25,26). Additionally synthesized esters with prolonged alkyl ester alcohols, e.g., the pentyl ester of AnA, exhibited only a similar antiandrogenic effect to the methyl ester MA (data not shown), intending that long alkyl side chains are obstructive for AR inhibition.

A very important issue for new drugs is their target specificity. To analyze whether the five AnA esters are functionally AR specific, the closely AR-related steroid receptors GR and PR-B, the more active PR isoform, were employed. Notably, none of the AnA esters affected the GR-mediated transactivation significantly (Figure 6A), whereas E1 and E3 only slightly inhibited the PR-B-mediated transactivation (Figure 6B). Nevertheless, the inhibition of the PR-B through E1 and E3 is much weaker compared to their inhibitory role for AR. These findings suggest that MA and E1-E4 are AR selective.

Furthermore, the inhibitory potency of the four new ester derivatives (E1-E4) was tested for the AR-T877A mutant. Even though the derivatives were added tenfold less concentrated, they inhibited the AR-T877A more potently compared to treatment with MA (Figure 6C). This indicates that the esters E1-E4 also have the advantageous potency to decrease AR-T877A-mediated transactivation.

To investigate the AR inhibition of endogenously expressed AR, E1-E4 were tested for their effect on the expression of the AR target

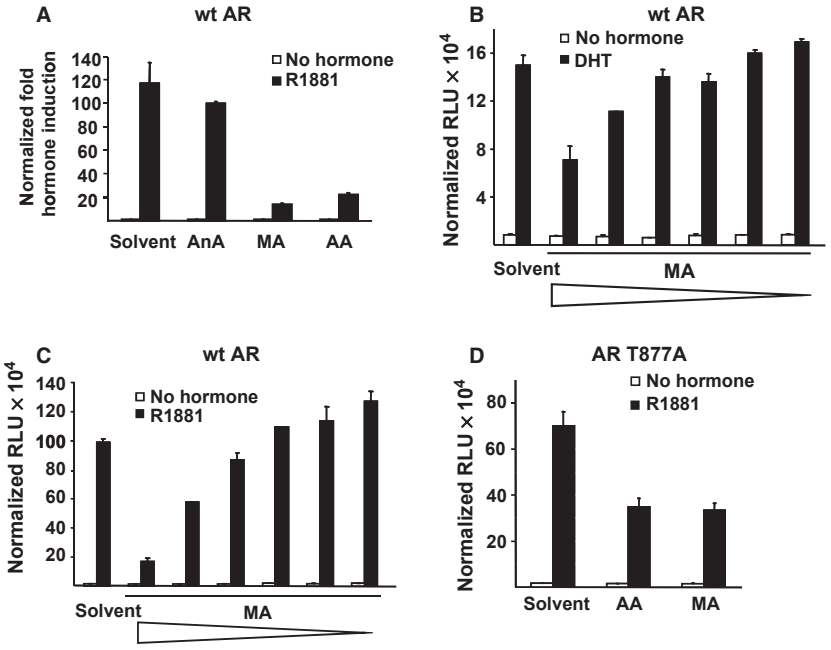


Figure 2: MA inhibits both the human wt androgen receptor (AR) and the T877A mutant in a concentration-dependent manner. For description of the test system, see Figure 1. Solvent (DMSO) was used as negative and the known antiandrogen atraric acid (AA, 10 μM) (18) as positive control. Error bars indicate the deviation of the mean of two wells. The experiments have been repeated at least three times. (A) MA inhibits the human wt AR. The *S. repens*-derived compound AnA (1000 μM) and its methyl ester MA (300 μM) were analyzed for their potency to inhibit the human AR activated by R1881 (30 pM). Each of the non-hormone-treated normalized values was set as one representing the normalized fold hormone induction is shown. (B) Concentration-dependent inhibition of dihydrotestosterone (DHT) activated human wt AR. The concentrations of 300 μM, 100, 30, 10, 3, and 1 μM of MA were used for the inhibition of human wt AR without hormone (white bars) or with DHT (50 nM) (black bars). (C) Concentration-dependent inhibition of R1881 activated human wt AR. The concentrations of 300, 100, 30, 10, 3, and 1 μM of MA were used for the inhibition of human wt AR without hormone (white bars) or activated by R1881 (30 pM) (black bars). (D) MA inhibits the AR T877A mutant. MA (300 μM) was analyzed for its potency to inhibit the AR T877A mutant without hormone (white bars) or activated by R1881 (black bars).

gene PSA with qRT-PCR in LNCaP cells. The concentrations used for E1-E4 were tenfold lower compared with MA. β-actin mRNA levels were determined and used for internal normalization. In line with the previous data, all derivatives including MA inhibit the AR-mediated endogenous PSA expression (Figure 6D and Supporting Information). Former studies showed that the AR is recruited to the PSA promoter in LNCaP cells, even if no androgen is present (14,27–29). Surprisingly, the repression without added hormone was as strong or even stronger compared to that with hormone, except in case of E1 (Figure 6D and Supporting Information), which may be attributed to residual AR activity mediated in a hormone-independent manner (28,29). When no hormone was added, the most potent derivative was E2 (Supporting Information), and if hormone was added, compound E1 showed the most potent inhibition of the PSA gene expression (Figure 6E). Taken together, the synthesized AnA derivatives exhibit more potency compared to MA.

Like MA, the derivatives E1-E4 do require the AR-LBD for the inhibition of the AR transactivation (data not shown). To investigate whether the AnA ester derivatives bind to the AR, a competitive whole cell binding assay was performed. As exemplary substance, E4 was used. Cos-7 cells that do not contain steroidal receptors were transfected with the human wt AR. The cells were treated with ^{3}(H)-mibolerone and either increasing concentrations of non-labeled mibolerone or E4. The AnA derivative E4 also induces the competition with the ^{3}(H)-mibolerone for AR binding, with a 50% displacement at a concentration of 10 μM, which confirms the IC_{50} mentioned above with being only slightly superior of those of currently therapeutically used AR antagonists (25,26). Thus, this coincides with the results of the reporter assay and suggests a direct binding of the AnA ester derivative E4 to the AR and simultaneously replacing the bound androgen.

AnA ester derivatives inhibit the proliferation of human androgen-dependent PCa cells (LNCaP)

The AR is a major regulator of PCa cell proliferation. To investigate whether MA and E1-E4 in fact can exert influence on the growth of human PCa cells, we performed cell growth assays with human androgen-dependent LNCaP cells by treating them for 11 days with the derivatives in medium containing 5% non-treated FCS (14,27,28). Solvent was used as negative control, and the antiandrogen atraric acid (AA) was taken as positive control (18). Cells were counted on the indicated days (Figure 7). Interestingly, inhibition of LNCaP cell proliferation can be seen for all added

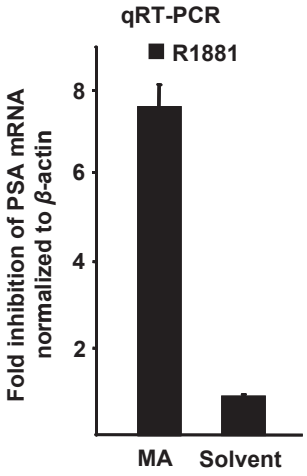


Figure 3: Inhibition of endogenous prostate-specific antigen (PSA) gene expression by the treatment of human LNCaP cells with MA. Quantitative real-time RT-PCR (qRT-PCR) experiments were performed for PSA mRNA and β-actin mRNA for normalization. The androgen-dependent LNCaP cells were grown in charcoal-stripped medium and treated with R1881 (30 pM) and with or without MA (300 μM) for 2 days. As negative control, solvent (DMSO) alone was used. Error bars indicate the deviation of the mean of two PCR samples. The experiments have been repeated four times.

ester derivatives comparing cell numbers to negative control. E4 showed the strongest effect on reducing the growth of PCa cells, suggesting E4 acts as a very potent PCa cell growth inhibitor. Neither apoptosis nor toxicity could be observed during derivative treatment.

Summarizing the data, our investigations resulted in the discovery of AnA ester derivatives as novel antiandrogenic active substances. Extensive analyses of five AnA esters showed that they can act as potent antiandrogens for both the wt AR and the T877A mutant. They also inhibit endogenous AR-mediated PSA expression and reduce LNCaP cell growth. Among the tested AnA derivatives are three novel molecules (E2-E4). Our data suggest that AnA esters provide a novel chemical lead structure for new antiandrogens and inhibitors of human PCa cell growth.

Discussion

In our study, we first discovered the antiandrogenic potential of AnA ester derivatives derived from AnA, which naturally occurs in saw palmetto fruits. In addition to the methyl ester MA, we synthesized four AnA esters (E1-E4) including three new structures (E2-E4) and investigated extensively their antiandrogenic potential. MA inhibits the AR-mediated transactivation at a concentration of 300 μM significantly. We found that the antiandrogenic activity of MA can be strongly enhanced by side-chain modifications (E1-E4). An elongation and branching of the ester alcohol (E1) results in a stronger antiandrogenic effect. Further variation of the ester alcohol combined with a single methylated amino group resulted in ester derivatives (E2-E4) with at least equal functional AR inhibition compared to E1. AnA esters E1-E4 are tenfold more potent than MA and inhibit the AR transactivation still significantly at a concentration of 30 μM or less. The conclusion that the antiandrogenic effect only results from increased lipophilicity because of an ester structure fails as we generated ester derivatives with different ester alcohols but with similar lipophilicity and observed great differences in their antiandrogenic potential (data not shown), suggesting that the ester alcohol residue is involved in antiandrogenic effectiveness. Previously, investigations in rats and rabbits showed that a considerable proportion of an orally administered AnA ester reaches the bloodstream unchanged, suggesting that these esters are stable (30).

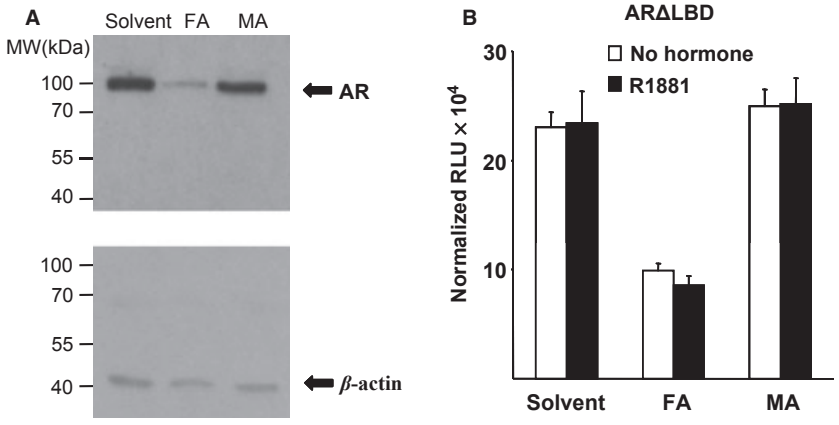
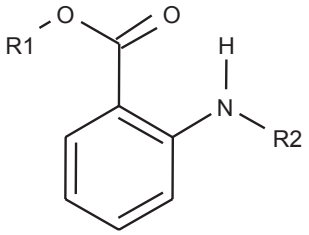


Figure 4: The AR-LBD is essential for MA-mediated inhibition of androgen receptor (AR). (A) MA does not affect the AR protein level in prostate cancer cells. The androgen-dependent prostate cancer cells LNCaP treated with MA for 3 days. Western blotting experiments were performed to detect the human wt AR. As negative control the treatment with solvent (ethanol) and as positive control flufenamic acid (FA) are indicated. Detections of β-actin levels served as internal control. (B) The ligand-binding domain is target for MA-mediated inhibition. The AR deletion mutant lacking the LBD (ARΔLBD) was tested for its ability to be repressed by MA in an experimental setup as described in Figure 1. Cells were treated with MA, as negative control solvent (ethanol) and as positive control FA were used.

Table 1: Overview of the investigated compounds



R1	R2	Compound Name	Symbol
-H	-H	Anthranilic acid	AnA
-CH3	-H	Methyl anthranilate	MA
-CH(CH3)CH2CH3	-H	Butan-2-yl-2-aminobenzoate	E1
-CH(CH3)CH2CH3	-CH3	Butan-2-yl-2-methylaminobenzoate	E2
-CH2CHCH2	-CH3	Prop-2-enyl-2-methylaminobenzoate	E3
-CH(CH3)CH2OCH3	-CH3	1-Methyoxypropan-2-yl-2-methylaminobenzoate	E4

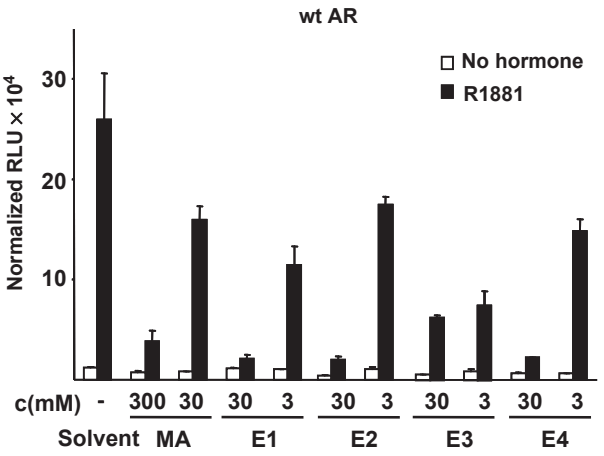


Figure 5: A variety of Anthranilic acid ester derivatives exhibit enhanced antiandrogenic effects. The indicated concentrations of compounds E1-E4 were analyzed for their potency to inhibit the human wt androgen receptor without hormone (white bars) or activated by R1881 (30 pM) (black bars) in an experimental setup as described in Figure 1.

Notably, all AnA ester derivatives inhibit the AR-T877A mutant. This mutant is found in several refractory PCa samples (5) particularly in hormone refractory PCa, which is very difficult to treat. It is fatal that the active metabolite of the currently used antiandrogen flutamide acts agonistic upon the AR-T877A resulting in an increased tumor growth.

Importantly, the esters did not impair the transactivation of the close AR relative to GR. The NHR PR-B is slightly targeted by E1 and E3 but compared with the AR inhibition, it is only an extremely weak effect.

As a further indication of AR inhibition, we found the mRNA amount of the AR target gene PSA decreasing by treating LNCaP cells with the esters. When no androgen was present, this inhibition could also be detected, surprisingly mostly higher compared to the androgen added samples. The reason might be attributed to the fact that the AR in LNCaP cells can bind to the PSA promoter in the absence of androgen (27). If so, our ester derivatives are able to inhibit the AR that is bound to the PSA gene regulatory regions, resulting in a decrease in the PSA mRNA level. In competitive whole cell binding studies, E4 indicated AR binding and thus it is likely that also the other AnA ester derivatives inhibit the AR by replacing the bound androgen.

In accordance with these results, all ester derivatives reduced the growth of the AR-T877A mutant expressed in LNCaP cells significantly. E4 even abrogated the growth of the LNCaP cells and therefore appears to be a very potent PCa cell growth inhibitor. The ester derivatives E1-E3 as well as MA are in the same range of cell growth inhibition as the positive control AA. We know that AA inhibits PCa cell growth AR dependently as the proliferation of PCa cells lacking the AR (PC3) cannot be inhibited by AA treatment (18). E4, in contrast, shows a very strong inhibition of LNCaP proliferation in the range of the error bar, resulting in a nearly complete block of cell proliferation. This suggests that MA as well as the compounds E1-E3 also inhibits PCa cell growth AR dependently, whereas E4 may have an additional AR-independent effect on PCa cell proliferation.

Studies investigating the concentration of OH-Fl in the plasma after oral application of Fl to PCa patients and healthy men revealed very high plasma concentrations (up to 8.5 μM) (31). The normal dosage of the therapeutically used antihormone Fl is about 250 mg (corresponding to 150 μM application for an 80-kg men) several times per day and the same concentration was also applied in this study (31).

Furthermore, our data reveal that the AR inhibition of AnA esters is not because of AR degradation on the protein level. Also corepressor interaction assays with the known AR corepressor Alien (27) revealed no measurable recruitment of Alien (Supporting Information).

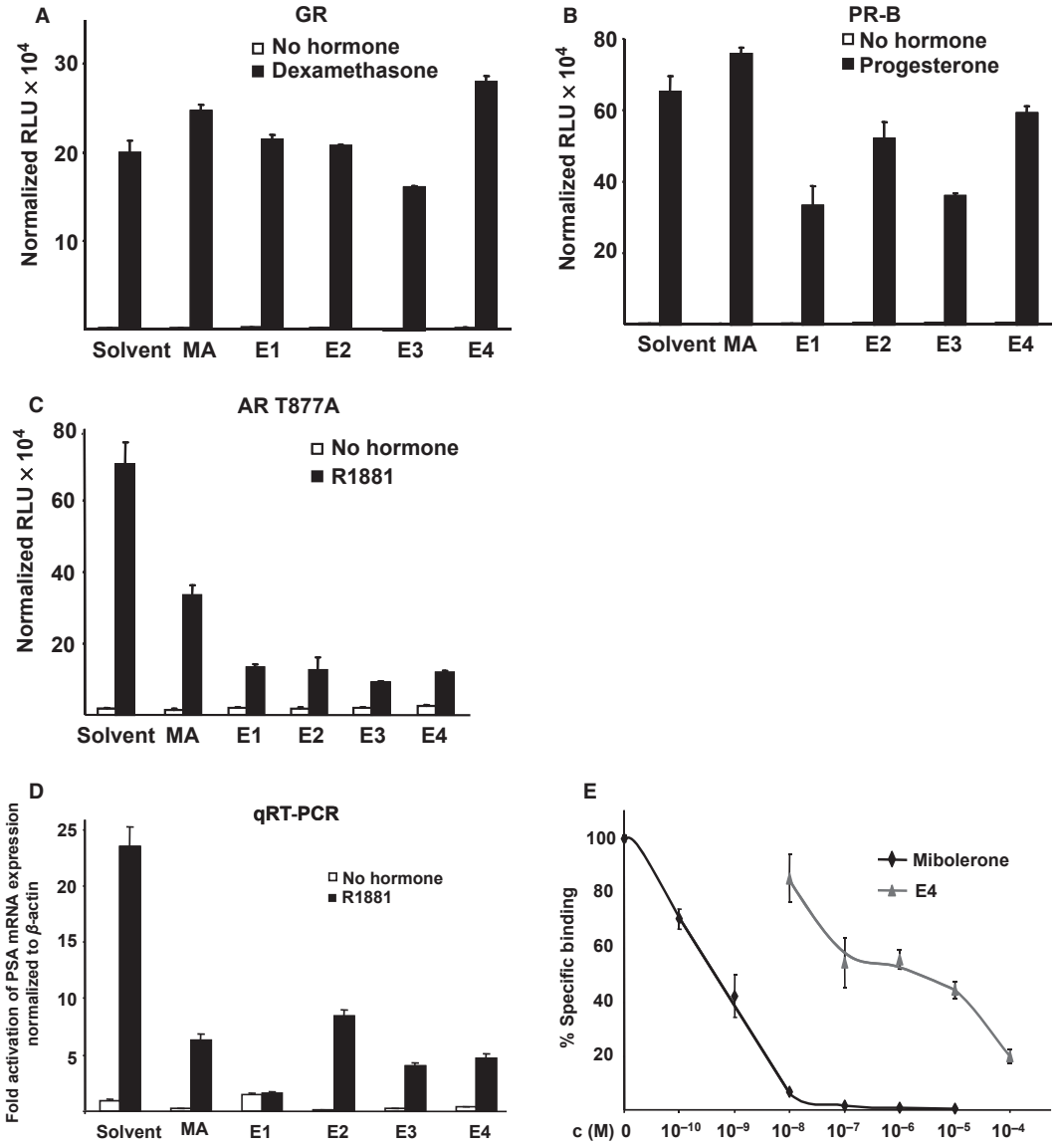


Figure 6: Anthranilic acid ester derivatives inhibit specifically the wt androgen receptor (AR) and the T877A mutant and endogenous prostate-specific antigen (PSA) gene expression. (A) MA and none of the derivatives E1-E4 inhibit significantly the GR. MA (300 μM) and E1-E4 (30 μM) were tested for their influence on the NHR GR without hormone (white bars) or activated by dexamethasone (1.8 nM) (black bars) in an experimental setup as described in Figure 1. (B) Influence of MA and E1-E4 on PR-B. The compounds MA (300 μM) and E1-E4 (30 μM) were tested for their activity on the NHR PR-B without hormone (white bars) or activated by progesterone (1 nM) (black bars) in an experimental setup as described in Figure 1. (C) The derivatives E1-E4 inhibit the AR T877A mutant. E1-E4 (30 μM) were analyzed for their potency to inhibit the AR T877A mutant without hormone (white bars) or activated by R1881 (30 pM) (black bars) in the experimental setup described in Figure 1. (D) Inhibition of endogenous PSA gene expression by MA and E1-E4 in the absence (white bars) and presence (black bars) of R1881 (1 nM). Quantitative real-time RT-PCR experiments were performed for PSA mRNA and β-actin mRNA detection to analyze the potency of MA and E1-E4 to inhibit PSA expression. The androgen-dependent LNCaP cells were grown for 2 days in charcoal-stripped medium and treated with the indicated substance for 36 h. The final concentration of MA was 300 and 30 μM for E1-E4. As negative control, solvent (DMSO) was used solely. Indicated is the fold activation where the RNA expression of the negative control containing solvent and in the absence of hormone is set as one. β-actin mRNA levels were used for normalization. Error bars indicate the standard deviation of the mean of two PCR setups. (E) E4 competes with androgens for AR binding. Competitive whole cell binding assays were performed using pSG5-hARwt-transfected COS-7 cells incubated with 1 nM ^{3}(H)-mibolerone in the absence and presence of increasing concentrations of either unlabeled mibolerone (diamond) or E4 (triangles) for 90 min. Competition for binding is illustrated by the percent of ^{3}(H)-mibolerone specifically bound to the AR. Results are averages of triplicates (±SEM).

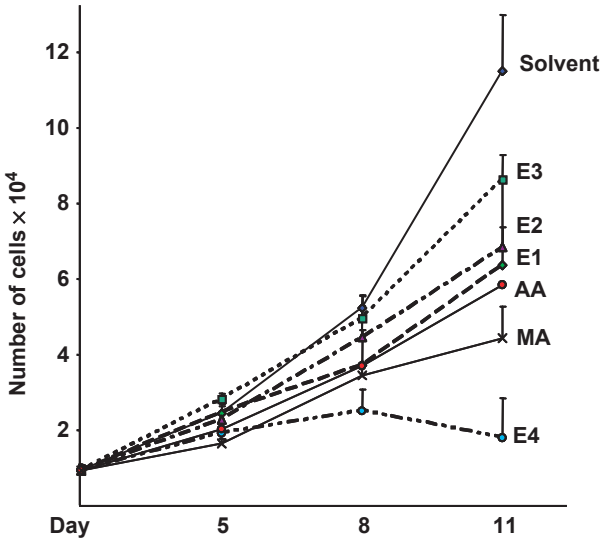


Figure 7: MA and E1-E4 inhibit the growth of the human PCa cell line LNCaP. LNCaP cells were treated with 300 μM MA or 30 μM E1-E4. Equal amounts of cells were seeded out in 5% untreated fetal calf serum containing medium and treated with the indicated derivatives. Solvent (DMSO) alone was used as negative and AA (10 μM) as positive control. The number of cells was counted at the indicated days (0, 5, 8, and 11). Error bars indicate the standard deviation of the mean of four wells.

Because MA is currently used as aromatic substance in several foods, its toxicity has been widely investigated. The WHO determined an ADI value of 1.5 mg/kg/day[a]. Pharmacological experiments using rats found that doses up to 150 mg/kg/day cause no toxic effects (32,33). The ADME properties (absorption, distribution, metabolism, and excretion) of propyl anthranilate were investigated in rats and rabbits *in vivo*. The biggest part of oral-administered propyl anthranilate was absorbed in the stomach. Only 7% reaches the small intestine (34). Besides only a very small part of the ester is hydrolyzed during the absorption process, whereas the biggest part (84%) of the ester reaches the blood circulation unchanged (34). Together, the facts that AnA esters seem to be stable esters *in vivo* (5), that MA has already been toxicologically studied with good tolerance (30), and that AnA esters can be easily synthesized render them to promising antiandrogenic drug candidates in the treatment of androgen-dependent diseases especially in PCa.

Conclusions

Altogether we synthesized potent AR antagonists by taking the natural compound AnA from saw palmetto fruits extracts as basic structure. All ester derivatives reveal AR-specific inhibition and repression of PCa cell growth, whereas they are novel or only less described compounds. In contrast to the currently used antiandrogens, the AnA esters likely do not recruit the AR corepressor Alien, also inhibit the hormone-therapy-resistant AR mutant, and thus presumably have another molecular mechanism for AR inhibition. Therefore, AnA ester derivatives can be a suitable basic structure to develop potent active substances as AR antagonists.

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Notes

[a]World Health Organization: Toxicological Evaluation of Some Flavouring Substances and Non-Nutritive Sweetening Agents. FAO Nutrition Meetings No. 44A, available at: http://www.inchem.org/documents/jecfa/jecmono/v44aje27.htm.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Subjacent reaction mechanism of the synthesized Esters E1-E4 and an overview of the structures of the analyzed compounds.

Figure S2. MA, E1-E4 inhibit the human wt AR in a dose dependent manner.

Figure S3. Inhibition of endogenous PSA gene expression by MA and E1-E4.

Figure S4. The AR inhibition by MA and E1-E4 does not involve the recruitment of the corepressor Alienα.

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