Spongian Diterpenoids Inhibit Androgen Receptor Activity

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Abstract

Androgen receptor is a ligand-activated transcription factor and a validated drug target for all stages of prostate cancer. Antiandrogens compete with physiologic ligands for androgen receptor ligand-binding domain (LBD). High-throughput screening of a marine natural product library for small molecules that inhibit androgen receptor transcriptional activity yielded the furanoditerpenoid spongia-13(16),-14-dien-19-oic acid, designated terpene 1 (T1). Characterization of T1 and the structurally related semisynthetic analogues (T2 and T3) revealed that these diterpenoids have antiandrogen properties that include inhibition of both androgen-dependent proliferation and androgen receptor transcriptional activity by a mechanism that involved competing with androgen for androgen receptor LBD and blocking essential N/C interactions required for androgen-induced androgen receptor transcriptional activity. Structure–activity relationship analyses revealed some chemical features of T1 that are associated with activity and yielded T3 as the most potent analogue. In vivo, T3 significantly reduced the weight of seminal vesicles, which are an androgen-dependent tissue, thereby confirming the on-target activity of T3. The ability to create analogues of diterpenoids that have varying antiandrogen activity represents a novel class of chemical compounds for the analysis of androgen receptor ligand-binding properties and therapeutic development.

Introduction

Localized prostate cancer is effectively managed by surgery, radiation, or active surveillance. Unfortunately, as many as 47% of these patients will experience recurrence and require systemic therapy for effective management of advanced disease (1). Generally, androgen ablation therapy by either chemical or surgical castration is provided to these patients to reduce levels of testicular androgen. After an initial and effective response to castration, resistance will inevitably occur with the development of lethal castration-resistant prostate cancer (CRPC). Despite of reduced levels of testicular androgen, there is compelling evidence to support a role of androgen receptor (AR) in CRPC (2).

Androgen receptor functions as a ligand-activated transcription factor that regulates gene expression in response to androgen. Binding of androgen to the ligand-binding domain (LBD) of the androgen receptor initiates a series of events that involve conformational changes that promote the dissociation of androgen receptor from HSPs and chaperones, resulting in phosphorylation, nuclear translocation, and androgen receptor dimerization. In the nucleus, androgen receptor directly binds to specific DNA sequences known as androgen response elements (ARE) that are present in the regulatory regions of androgen-regulated genes and DNA-bound androgen receptor recruits co-activators and assembly of transcriptional machinery to initiate transcription. Genes regulated by androgen receptor contribute to proliferation and survival of prostate cancer (3). Thus, expression and function of androgen receptor are essential for proliferation and growth of prostate cancer cells (4). Importantly, androgen receptor signaling remains functionally active in CRPCs (5, 6). Molecular mechanisms suspected to be involved in the continued androgen receptor activity in CRPC include (i) androgen receptor gene amplification and/or increased expression of androgen receptor (7–9), (ii) gain-of-function mutations in androgen receptor that result in activation by nonandrogenic steroidal ligands and antiandrogens (10, 11), (iii) expression of constitutively active androgen receptor splice variants that lack ligand-binding domain (12), (iv) ligand-independent activation of androgen receptor by alternative pathways such as the cAMP-dependent protein kinase, interleukin-6, and other factors (13–17), (v) increased expression of androgen receptor coactivators (18–21), and (vi) intratumoral de novo synthesis of androgens (22). Together...
these findings suggest that targeting androgen receptor is a viable approach for clinical management of all stages of prostate cancer including CRPCs.

Androgen receptor is targeted indirectly by androgen ablation therapy that reduces androgen that binds to the androgen receptor LBD. LHRR analogues, orchitectomy, and inhibitors of androgen synthesis are standard approaches used clinically to reduce levels of androgen. Abiraterone is an irreversible inhibitor of CYP17 that is involved in androgen synthesis. Abiraterone increases survival by 3.9 months in patients with CRPCs who have previously failed androgen ablation and docetaxel therapies (23). Antiandrogens competitively bind to androgen receptor LBD to antagonize the action of androgens and thereby attenuate androgen receptor transcriptional activity. Nonsteroidal antiandrogens used clinically for prostate cancer include bicalutamide (BIC), flutamide, nilutamide, and enzalutamide (MDV3100). The phase III AFFIRM trial showed that enzalutamide has a median overall survival advantage of 4.8 months compared with placebo in patients with CRPCs post-docetaxel treatment (24). Despite of the survival benefits of a potent antiandrogen such as enzalutamide, all antiandrogens ultimately fail. However, once an antiandrogen fails, changing to an alternative second-line antiandrogen can be clinically effective with improved survival (25, 26), thereby supporting the quest to discover additional antiandrogens for the clinical management of CRPCs. Here, we report that the furanoditerpenoid spongia-13(16),14-dien-19-oic acid (T1) and the 2 semisynthetic derivatives T2 and T3 are antiandrogens.

Materials and Methods
Cell lines, proliferation assay, and transfection for luciferase assay
LNCaP human prostate cancer cells were maintained in RPMI-1640 supplemented with 10% (v/v) FBS (Invitrogen by Life Technologies). PC3 cells were maintained in Dulbecco’s Modified Eagle Media (DMEM) with 5% (v/v) FBS. CV-1 monkey kidney cells were maintained in MEM with 10% (v/v) FBS and 1% L-glutamine. VCaP cells were maintained in RPMI-1640 containing 10% (v/v) FBS (Invitrogen). We obtained the cell line derived from the parental LNCaP cells, were maintained in culture no more than 10 to 15 passages studies conducted on these cells (27). All cells are maintained in culture no more than 10 to 15 passages studies conducted on these cells (27). All cells are maintained in culture no more than 10 to 15 passages studies conducted on these cells (27). All cells are maintained in culture no more than 10 to 15 passages studies conducted on these cells (27). All cells are maintained in culture no more than 10 to 15 passages studies conducted on these cells (27). All cells are maintained in culture no more than 10 to 15 passages studies conducted on these cells (27). All cells are maintained in culture no more than 10 to 15 passages studies conducted on these cells (27). All cells are maintained in culture no more than 10 to 15 passages studies conducted on these cells (27). All cells are maintained in culture no more than 10 to 15 passages studies conducted on these cells (27). All cells are maintained in culture no more than 10 to 15 passages studies conducted on these cells (27). All cells are maintained in culture no more than 10 to 15 passages studies conducted on these cells (27). All cells are maintained in culture no more than 10 to 15 passages studies conducted on these cells (27). All cells are maintained in culture no more than 10 to 15 passages studies conducted on these cells (27). All cells are maintained in culture no more than 10 to 15 passages studies conducted on these cells (27). All cells are maintained in culture no more than 10 to 15 passages studies conducted on these cells (27). All cells are maintained in culture no more than 10 to 15 passages studies conducted on these cells (27). All cells are maintained in culture no more than 10 to 15 passages studies conducted on these cells (27). All cells are maintained in culture no more than 10 to 15 passages studies conducted on these cells (27). All cells are maintained in culture no more than 10 to 15 passages studies conducted on these cells (27). All cells are maintained in culture no more than 10 to 15 passages studies conducted on these cells (27). All cells are maintained in culture no more than 10 to 15 passages studies conducted on these cells (27). All cells are maintained in culture no more than 10 to 15 passages studies conducted on these cells (27). All cells are maintained in culture no more than 10 to 15 passages studies conducted on these cells (27).

Endogenous expression of androgen-regulated genes
LNCaP cells (180,000 cells/well) in 6-well plates were incubated for 48 hours in serum-free RPMI before pre-treatment for 1 hour with dimethyl sulfoxide (DMSO) vehicle or small molecules at 10 μmol/L before addition of 1 nmol/L R1881. VCaP cells (300,000 cells/well) were plated in 6-well plates in DMEM with 5% dextran-coated charcoal-stripped serum. Two days later, small molecules and R1881 were added to VCaP cells in the same manner as LNCaP. Total RNA was isolated after 48 hours (for LNCaP) and 16 hours (for VCaP) by using RNEasy Micro Kit (QIAGEN) and subsequently reverse transcribed to cDNA by SuperScriptIII First-Strand Synthesis System for RT-PCR (Invitrogen). Diluted cDNA and gene-specific primers were combined with Platinum SYBR Green qPCRSuperMix-UDG with ROX (Invitrogen), and the transcripts were measured by quantitative real-time (qRT)-PCR (ABI PRISM, Applied Biosystems by Life Technologies). qRT-PCR was carried out separately in triplicates for each biologic sample. Expression levels were normalized to housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers used were previously described (28–31). Sequence of primers used is listed: GAPDH: F: 5’-CTGAACCTCAACGCGA-CACC-3’ and R: 5’-TGCTGTTAGCCTTGTGTT-3’; PSA: F: 5’-CGGATTTACGTGCTCTGCT-3’ and R: 5’- CCCATGACGTGATACCTTGA-3’; KLK2: F: 5’-TGTTGCTA GAG CT ACT CTG A-3’ and R: 5’-CCA CTT CCG GTA ATG CAC CA-3’; FKBP5: F: 5’-CGACAGATA-TACGCCCCATCT-3’ and R: 5’-CAAGTCTTCTGCCC-CATTGC-3’; TMPRSS2: F: 5’-GGACAGTGTGCACTCCTGAA-A-3’ and R: 5’-TCCAGGAGAAGGTTCC-3’; and AR: F: 5’-CCA TCTGTTGCTCTGGAAATGT-TAT-3’ and R: 5’-AGCTTCTGGTTGTCTCTCAGTG-G-3’.

In vitro ligand-binding assay
Androgen receptor, progesterone receptor, and estrogen receptor-α PolarScreen Competitor Assay kits (Invitrogen) were used according to the manufacturer’s protocol. Serial dilution was done for each small molecule and solvent was compensated to ensure equal volume of DMSO and ethanol in each sample. Fluorescence polarization at excitation wavelength 470 nm and emission at 535 nm were measured in Greiner 384 black clear bottom plates by using Infinite M1000 (TECAN).

Animal studies
Twelve-week-old male NOD/SCID intact mice were maintained in the Animal Care Facility at the British Columbia Cancer Agency Research Center. All animal experiments were approved by the University of British Columbia Animal Care Committee and strictly followed the ethical regulatory standards. Mice were divided into 3 groups: DMSO control (n = 10), bicalutamide (n = 8), and T3 (n = 8). BIC and T3 were administered by oral
Diterpenoids Are Antiandrogens

Diterpenoids inhibit androgen receptor transcriptional activity

The chemical structures, together with the observed reduction of androgen-dependent proliferation of androgen receptor–positive LNCaP cells, suggest that T1 and T3 may be inhibitors of androgen receptor. To test this hypothesis, the effects of diterpenoids on the transcriptional activity of endogenous androgen receptor in LNCaP cells were tested using the androgen receptor–driven ARR3-LUC reporter gene construct. Androgen-induced ARR3-LUC reporter activity was potently blocked to approximately baseline levels by T1 and T3 at 10 μmol/L concentration and this inhibition was comparable with that achieved with an equal concentration of bicalutamide (Fig. 2A). Interestingly, 10 μmol/L T2 also significantly inhibited androgen receptor transcriptional activity although it was less potent than bicalutamide, T1, or T3 with only 50% inhibition. Dose–response curves (Fig. 2B) estimated the IC50 values for inhibition of endogenous androgen receptor transcriptional activity in LNCaP cells to be 0.21 μmol/L for bicalutamide, 4.2 μmol/L for T1, 0.41 μmol/L for T3, and greater than or equal to 10 μmol/L for T2. LNCaP95 cells, an androgen-independent subline of LNCaP cells, has a functional androgen receptor that can be activated by androgen but its proliferation is not altered by androgen (27). R1881 weakly induced ARR3-LUC reporter activity in LNCaP95 cells by approximately 4-fold over control levels, which was significantly attenuated by bicalutamide, T1, and T3 (Supplementary Fig. S3A). T2 had no significant effect on androgen-induced ARR3-LUC activity in this cell line. LNCaP95 cell growth/viability was not altered by R1881, bicalutamide, or any of the diterpenoids (Supplementary Fig. S3A). Together, these data reveal that these diterpenoids were effective in blocking transcriptional activity of endogenous androgen receptor in LNCaP cells with T2 being less potent than T1 and T3.

Endogenous androgen receptor in LNCaP cells has the T877A mutation in the androgen receptor LBD (AR-T877A) that alters both ligand specificity and affinity (34). Therefore, to investigate the effect of diterpenoids on wild-type androgen receptor transcriptional activity, CV1 cells that do not express endogenous androgen receptor were transiently cotransfected with ARR3-LUC reporter and an optimized amount of an expression vector for wild-type human androgen receptor that can be activated by androgen but whose proliferation is not altered by androgen (27). R1881 weakly induced ARR3-LUC reporter activity in CV1 cells by approximately 32-fold over control levels, which was significantly attenuated by bicalutamide, T1, and T3 (Supplementary Fig. S1). T2 had no significant effect on androgen-induced ARR3-LUC activity in this cell line. CV1 cell growth/viability was not altered by R1881, bicalutamide, or any of the diterpenoids (Supplementary Fig. S3A). Together, these data reveal that these diterpenoids were effective in blocking transcriptional activity of endogenous androgen receptor in CV1 cells with T2 being less potent than T1 and T3.

Results

Diterpenoids reduce androgen-dependent proliferation of prostate cancer cells

Diterpenoids T1, T2, and T3 share an unsubstituted tricylic perhydrophenanthrene ABC ring system fused to a 5-membered D ring with the physiologic androgen receptor ligand dihydrotestosterone (DHT) as well as the synthetic androgen R1881 (Fig. 1A). Bicalutamide is a nonsteroidal antiandrogen used clinically to block androgen receptor activity and subsequent androgen-dependent proliferation of prostate cancer cells (32). To evaluate T1, T2, and T3 on androgen-dependent proliferation, LNCaP human prostate cancer cells that express functional full-length androgen receptor, although mutated in the LBD, were treated with vehicle, or 10 μmol/L of bicalutamide (positive control), T1, T2, or T3. As expected, 0.1 nmol/L of R1881 led to increased proliferation in LNCaP cells (Fig. 1B). Both T1 and T3 inhibited androgen-dependent proliferation with T1 being significantly more effective than T3 and comparable with BIC whereas T2 had no effect (Fig. 1B). To determine the specificity of T1 and T3 for attenuating androgen receptor–dependent proliferation, PC3 human prostate cancer cells were used because these cells do not express a functional androgen receptor. Consistent with PC3 cells not being dependent on androgen receptor activity for growth, 0.1 nmol/L R1881 did not alter their proliferation. Importantly, none of the diterpenoids inhibited proliferation of these cells (Fig. 1C), suggesting that these diterpenoids are not generally toxic. PC3 cells treated with each diterpenoid showed increasing proliferation over 24-, 48-, and 72-hour time points with no difference as compared with the vehicle control (Supplementary Fig. S1). Microscopic analysis of LNCaP cells exposed to 10 μmol/L T1, T2, or T3 for 4 days also revealed no signs of cytotoxicity (Fig. 1D). Viability assays showed no indication of cytotoxicity in LNCaP, VCaP that have a wild-type androgen receptor, or PC3 cells treated for 4, 5, and 3 days, respectively, with diterpenoids or bicalutamide (Supplementary Fig. S2). These viability assays mirrored proliferation data shown here and were consistent with previous studies that androgen-dependent proliferation of VCaP cells that express wild-type androgen receptor are poorly inhibited with bicalutamide (33). Together these data suggest that T1 and T3 may have specificity for cells that are dependent on androgen receptor for growth and that these diterpenoids are not generally cytotoxic.

gavage at 10 mg/kg daily for a total of 13 doses. Initial and final body weights were recorded for each animal. Mice were sacrificed 14 days after the first treatment, and tissues were collected and weighed. Prism (GraphPad Software) was used to generate whisker plots for the weight of seminal vesicles and testes. Change in body weight was calculated from the difference between initial and final weight as a percentage of initial weight. Student t test was conducted.
These IC$_{50}$ values were consistent with the trends observed for the potency of compounds on wild-type androgen receptor observed at the single concentration of 10 µmol/L (Fig. 2C). Importantly, the presence of a furan or the reduced tetrahyrofuran in the chemical structures of the diterpenoids appear to alter activities obtained with wild-type androgen receptor and mutant AR-T877A as measured for T1 and T2.

**Diterpenoids inhibit endogenous expression of androgen-regulated genes**

Androgen receptor regulates the transcription of hundreds of genes in prostate cells with several well-characterized genes, such as PSA, KLK2, FKBP5, and TMPRSS2, shown to have functional AREs (35–38). To test the effects of diterpenoids on endogenous expression of androgen-regulated genes, qRT-PCR was used to measure the levels of these transcripts in cells exposed to 10 µmol/L of each diterpenoid. First LNCaP cells with mutated androgen receptor were tested. Among the 3 compounds, T1 was consistently the most effective inhibitor of androgen-induced gene expression with a potency comparable with or better than bicalutamide (Fig. 3A). T3 also inhibited androgen-induced gene expression, whereas T2 showed no significant effects in blocking androgen-induced expression of this set of genes. Importantly, in the absence
of androgen, T1 and T2 decreased basal levels of transcripts, whereas T3 significantly increased basal expression, suggesting that T3 may be a partial agonist of mutated AR-T877A similar to bicalutamide (39).

Levels of transcripts of androgen-regulated genes are sensitive to changes in the levels of androgen receptor. Therefore, the levels of androgen receptor were measured in LNCaP cells treated with the diterpenoids. In the absence of androgen, levels of androgen receptor mRNA were significantly decreased by both T1 and T3 (Fig. 3B). The decrease in androgen receptor mRNA by T1 was consistent with the observed decrease in androgen receptor protein by this compound in the absence of androgen by a currently unknown mechanism (Fig. 3C). However, in the presence of androgen, the diterpenoids had no significant effect on androgen receptor levels, thereby suggesting that inhibition of androgen-induced transcription of androgen receptor–regulated genes is not a mechanism that involves decreased levels of androgen receptor.

Analysis of androgen receptor–regulated gene expression was next examined in VCaP cells that endogenously express wild-type androgen receptor. As expected, levels of PSA transcript were weakly increased in response to androgen (Fig. 3D) and neither bicalutamide nor any of the diterpenoids significantly reduced these levels. However, levels of transcript for FKBP5 and TMPRSS2 were robustly increased in response to androgen and both were significantly blocked by bicalutamide and T3 whereas T1 and T2 had no significant effect. Together, these data are consistent with the trends observed with wild-type androgen receptor using a reporter gene assay in CV1 cells (Fig. 2C).

**Figure 2.** Inhibition of androgen receptor transcriptional activity. A, LNCaP cells were transfected with ARR3-LUC and treated with 10 μmol/L of BIC, T1, T2, T3, or vehicle (VEH) in the absence or presence of 1 nmol/L R1881 (− or + ) for 48 hours. B, LNCaP cells transfected with ARR3-LUC were treated with diterpenoids at 10, 1, 0.1, and 0 μmol/L in the presence of 1 nmol/L R1881 for 48 hours. C, CV1 cells were cotransfected with pSV-AR0, the expression vector for wild-type androgen receptor, and ARR3-LUC before treatment with 10 μmol/L of BIC, T1, T2, T3, or VEH in the absence or presence of 1 nmol/L R1881 (− or + ) for 24 hours. D, CV1 cells transiently cotransfected with pSV-AR0 (wild-type androgen receptor) and ARR3-LUC were treated with diterpenoids at 10, 5, 2.5, 1, and 0 μmol/L in the presence of 1 nmol/L R1881 for 24 hours. Error bars represent the mean ± SEM, n = 3 independent experiments. Student t test: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Diterpenoids bind to androgen receptor LBD and inhibit androgen receptor N/C interaction

The chemical structures of diterpenoids resemble steroids, thereby suggesting that they may physically interact with androgen receptor LBD. To determine whether these diterpenoids bind the androgen receptor LBD and, if so, to assess their binding affinities to the androgen receptor LBD, we conducted in vitro ligand competitor assays using recombinant wild-type androgen receptor LBD. R1881 had strong affinity for androgen receptor LBD with a half maximal effective concentration (EC50) calculated at 5.3 nmol/L (Fig. 4A). T1 showed relatively weaker binding with an EC50 of 27.1 μmol/L, whereas T2 was much weaker at 398.4 μmol/L. However, T3 which has the carboxylic acid functionality present in T1 reduced to a primary alcohol displayed improved binding with an EC50 of 1.6 μmol/L, which was comparable with that achieved by bicalutamide at 1.1 μmol/L. EC50 values for diterpenoids on wild-type recombinant androgen receptor LBD were consistent with trends observed in cells with...
wild-type androgen receptor at 10 μmol/L diterpenoid (Fig. 2C) as well as the IC50 values for wild-type androgen receptor transcriptional activity (Fig. 2D).

Androgen receptor transcriptional activity in response to androgen requires interaction between the N-terminal domain and the C-terminus LBD of androgen receptor (N/C interaction; ref. 40). Antiandrogen such as bicalutamide inhibits androgen-induced N/C interaction (41). To determine whether diterpenoids also inhibit androgen-induced androgen receptor N/C interaction, the mammalian 2-hybrid system was employed using wild-type LBD. T1 and T3 significantly inhibited androgen-induced N/C interaction (Fig. 4B). Inhibition of N/C interaction by T3 was comparable with that achieved with BIC. T2 had no effect, which was consistent with its poor affinity for wild-type androgen receptor. Together, these data suggest that T1 and T3 inhibit N/C interaction of androgen receptor by directly binding to androgen receptor LBD.

**Specificity of diterpenoids**

Androgen receptor is a member of the steroid receptor superfamily, and its LBD shares substantial sequence identity with the LBDs of progesterone receptor (PR) and glucocorticoid receptor (GR) at 55% and 51%, respectively. Some antiandrogens such as bicalutamide are potent inhibitors of PR transcriptional activity (42). To test receptor specificity of diterpenoids for androgen receptor, the transcriptional activities of PR and GR were examined using their respective reporter gene constructs transiently
transfected into LNCaP cells, which do not endogenously express PR and GR. Induction of PR transcriptional activity by its ligand 4-pregnen-3,20 diene (Preg) was inhibited by bicalutamide and RU486, a potent PR inhibitor, as well as by T1 and T3, whereas T2 showed no significant effect (Fig. 5A). The 3 diterpenoids and bicalutamide had no effect on the transcriptional activity of GR induced by dexamethasone (Fig. 5B). These results suggest that diterpenoids have some specificity for androgen receptor and PR and that they do not have general inhibitory effects on transcription and translation. Consistent with T1 and T2 inhibiting PR transcriptional activity, these compounds competed for the PR LBD as shown using the in vitro ligand competitor assay with recombinant human PR LBD (Fig. 5C). RU486 had strong binding affinity to PR LBD with an EC50 of 45 nmol/L, comparable to Preg at 35 nmol/L, whereas bicalutamide and T1 had similar EC50 values at 5,100 and 5,600 nmol/L, respectively. T3 had a binding affinity to PR LBD at about 500 nmol/L, which agreed with its potent inhibition on PR transcriptional activity (Fig. 5A). T2’s EC50 could not be assessed due to low activity and poor solubility at high concentrations. Consistent with estrogen receptor α (ERα) having little sequence homology to androgen receptor, none of the androgen receptor ligands (R1881, bicalutamide, and the diterpenoids) were efficient in competing with fluoromone for ERα (Fig. 5D).

**In vivo effects of diterpenoid T3 on androgen-dependent tissue**

In vivo, blocking the androgen axis results in atrophy of androgen-dependent tissues such as the seminal vesicles and thereby provides an indication of on-target activity (43, 44). Of the 3 diterpenoids tested, T3 was consistently the most potent inhibitor of wild-type androgen receptor and was therefore chosen for in vivo evaluation for effects on benign tissue that would harbor wild-type androgen receptor. Mature male mice treated with T3 daily oral doses of T3 had a significant decrease in seminal vesicle weight (Fig. 6A), which was consistent with the properties of an antiandrogen. No changes in the testes weight (Fig. 6B) or body weight (Fig. 6C) were observed, thereby suggesting a relatively specific effect of T3 on androgen-dependent tissue as opposed to it merely being toxic.

**Discussion**

Androgen receptor is a ligand-activated transcription factor that plays an important role in prostate cancer. Drug development to block androgen receptor transcriptional activity for the treatment of this disease has yielded steroidal and nonsteroidal antiandrogens. Here, T1 was originally isolated from a crude extract of a marine sponge based upon its activity in our screen for inhibitors of androgen receptor transcriptional activity. T1 belongs to a family of compounds called spongian diterpenoids, which are commonly found in marine sponges and shell-less mollusks that feed on the sponges (45). T2 and T3 are semisynthetic compounds produced by reducing either the furan or 17β carboxylic acid functionalities in T1, respectively. Spongian diterpenoids have been previously investigated for in vitro antiviral and antitumor activities (46). This is the first report that diterpenoids have antiandrogen activity as shown by (i) inhibition of androgen-dependent proliferation while having no effect on cells that do not depend on androgen receptor for growth and survival, (ii) blocking androgen receptor transcriptional activity measured by reporter gene constructs and endogenous gene expression, (iii) specificity for androgen receptor with no effect on GR transcriptional activity and no effect on the proliferation of cells that do not express functional androgen receptor, (iv) competing with androgen for the LBD, (v) blocking androgen-induced N/C interaction, and (vi) reducing the weight of androgen-dependent tissue in vivo while having no effect on body weight. The mechanism of action of diterpenoids to inhibit androgen receptor transcriptional activity involved binding to the androgen receptor LBD as shown in vitro ligand competition binding assays. Consistent with other antiandrogens for blocking N/C
interaction induced by androgen (47), the diterpenoids also inhibited this interaction.

Androgen receptor mediates the effects of androgen, which is the major mitogen for prostate cancer, thereby providing the rationale for targeting androgen receptor for the treatment of prostate cancer. Here, furano diterpenoids T1 and T3 inhibited androgen-dependent proliferation of LNCaP human prostate cancer cells that are androgen-sensitive and express functional androgen receptor. The antiproliferative effect of T1 and T3 was not observed in PC3 cells that do not have a functional androgen receptor, thereby suggesting potential specificity of these diterpenoids for cells that are dependent on androgen receptor for growth and survival. Consistent

![Figure 5. Effects of diterpenoids on transcriptional activity of related steroid receptors.](image)

Figure 5. Effects of diterpenoids on transcriptional activity of related steroid receptors. A, LNCaP cells were transiently cotransfected with an expression plasmid for full-length human (PR) and a PR-driven (PRE) luciferase reporter. Cells were pretreated for 1 hour with 10 μmol/L of BIC, RU486, each diterpenoid, or vehicle (VEH) before the addition of 10 nmol/L Preg as indicated by black bars (+) or ethanol control (white bars and -). B, LNCaP cells were transiently cotransfected with an expression plasmid for full-length human GR and GR-driven (GRE) reporter and pretreated with 10 μmol/L of BIC, diterpenoids, or VEH before the addition of 10 nmol/L dexamethasone (Dex) as indicated by black bars (+) or ethanol control (white bars and -). 48 hours after treatment, luciferase assay was conducted and relative luminescent unit per minute was measured and normalized to protein concentration. Error bars represent the mean ± SEM, n ≥ 3 independent experiments. Student t test: *, P < 0.05; **, P < 0.01; ***, P < 0.001. Recombinant human PR LBD (C) and recombinant human full-length ERα (D) were tested for the binding affinity of the diterpenoids by measuring fluorescence polarization (mP) with an excitation wavelength of 470 nm and emission wavelength of 535 nm. Serial dilution was conducted for the testing compounds. A representative plot from at least 3 independent assays is shown for each receptor.

![Figure 6. Efficacy of diterpenoids in vivo.](image)

Figure 6. Efficacy of diterpenoids in vivo. Male NOD-SCID intact mice were treated with DMSO, BIC, or T3 at 10 mg/kg body weight for 2 weeks. Tissues were collected, and their weights were measured. The weight of seminal vesicle (A) and testes (B) were plotted as whisker plots. Body weight (C) was plotted as the percentage change between initial and final weights. Error bars represent the mean ± SEM, n = 8 to 10 as indicated. Student t test: *, P < 0.05; **, P < 0.01; ***, P < 0.001.
with this interpretation, administration of T3 to mature male mice reduced the weight of androgen-dependent seminal vesicles while having no effect on body weight. T3 had no effect on the weight of testis, similar to bicalutamide and consistent with other inhibitors of the androgen axis (48). Other data supporting specificity of the diterpenoids for blocking androgen receptor transcriptional activity include that they did not broadly inhibit transcription and translation or affect all steroid hormone receptors as indicated by a lack of effect on the transcriptional activity of the closely structurally related GR. However, similar to bicalutamide, T1 and T3 inhibited PR transcriptional activity whereas T2 did not. PR is not known to be associated with any essential biologic function in mature men and bicalutamide, a PR inhibitor, has been used clinically to treat prostate cancer for many years with an acceptable safety profile (32). Consistent with its relatively modest biologic activity, T2 showed very weak binding affinity to androgen receptor and PR. However, both T1 and T3 were shown to bind to androgen receptor and PR, T3 clearly showed stronger binding affinity, which was comparable to bicalutamide for androgen receptor. Together these data suggest these spongian diterpenoids are novel small-molecule inhibitors of androgen receptor with T3 having the best potency of the 3 compounds.

LNCaP cells have a mutated androgen receptor LBD (T877A), which reduces ligand specificity as well as alters ligand affinity and dissociation rates (34, 49). Thus, inhibitory properties of compounds that bind to androgen receptor LBD can be altered by mutations within this domain. When comparing the properties of the 3 diterpenoids on wild-type versus mutated AR-T877A, T1 activity was the most affected by this mutation. T1 had no effect on wild-type androgen receptor transcriptional activity with accompanying poor inhibition of N/C interaction and higher IC50 and EC50 values. However, with the mutated AR-T877A in LNCaP cells, T1 became a potent inhibitor of both androgen-dependent proliferation and androgen receptor transcriptional activity with an IC50 of 4.2 μmol/L. Generally, T2 had poor activity, whereas T3 had good activity regardless of whether the androgen receptor had the T877A mutation.

T3 was synthesized with the intention of maintaining the furan ring in T1 but modifying the 17β carboxylic acid group to determine whether a compound could be generated that had better binding affinity to androgen receptor and consequently be a more potent inhibitor. From in vitro ligand competition binding assays, T3 indeed showed approximately 16-fold higher affinity to androgen receptor LBD with an EC50 of 1.6 μmol/L than to T1 (27.1 μmol/L), making T3 comparable to bicalutamide (1.1 μmol/L). Improved affinity of T3 compared with T1 probably involves specific interactions with a set of well-conserved amino acid residues in the LBD of androgen receptor. Hydrophobic interactions between the perhydrophenanthrene skeleton of the ligand and the amino acid residues within the ligand-binding pocket are critical for binding as well as hydrogen-bonding which would impact affinity. Comparing with other nonsteroidal small-molecule inhibitors of the androgen receptor LBD such as bicalutamide and enzalutamide, the diterpenoids represent a novel class of chemical compounds with antiandrogen activity. Thus, further structure–activity relationship studies are ongoing with the intention of developing more potent derivatives of T1 and T3 with optimized drug-like qualities for their potential clinical application for prostate cancer and/or other diseases involving the androgen axis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: Y.C. Yang, G. Carr, M.D. Sadar
Development of methodology: Y.C. Yang, N.R. Mawji, G. Carr
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y.C. Yang, A.H. Tien, G. Carr, J. Wang, M.D. Sadar
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Synthesized analogues based on the lead compound and provided pure material for biologic testing: L.G. Meimetis

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