Identification of triptophenolide from Tripterygium wilfordii as a pan-antagonist of androgen receptor

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Identification of triptophenolide from *Tripterygium wilfordii* as a pan-antagonist of androgen receptor

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**KEYWORDS:** androgen receptor; triptophenolide; gene report system; traditional Chinese medicine.

**ABSTRACT:** A compound, triptophenolide, derived from *Tripterygium wilfordii* was identified as an antiandrogen. Triptophenolide inhibits the activity of both wild-type and F876L mutant ARs. Triptophenolide exhibits its anti-androgenic activity through competitive binding with androgen in the hormone-binding pocket, decreasing the expression of androgen receptor, and reducing the nuclear translocation of androgen receptor.

Prostate cancer (PCa) is one of the most common cancers and the second leading cause of cancer death in men of the western countries\(^1\). Androgen receptor (AR) plays a critical role in the development and progression of PCa\(^2\). Antiandrogens have become a main therapeutic agent for advanced PCa. Up to now, current clinically used antiandrogens such as flutamide (FLU), bicalutamide (BIC), and newly approved enzalutamide (ENZ) mainly target the hormone-binding pocket (HBP) of the AR ligand binding domain (LBD). These approved antiandrogens have greatly improved the survival and life quality of the PCa patients. However, after the initial effective response, most tumors develop resistance to the antiandrogens used for therapy due to the AR overexpression\(^3\), the AR mutants\(^4, 5\), or the bypassing pathway activation\(^6, 7\), etc. Therefore, it is increasingly urgent to seek for the new strategy to obtain novel antiandrogens in order to circumvent the developed resistance of PCa for the currently clinically used antiandrogens. On the other hand, traditional Chinese medicine (TCM) has a very long history in cancer therapy. To date, TCM is widely used as a complementary therapy for cancer and is found improving symptoms and the quality of life and even extend lifespan of cancer patients\(^8, 9\), thus providing a potential resource for therapeutic agents of prostate cancer. Importantly, one herb, *Tripterygium wilfordii*, apart from its application in the treatment of rheumatoid arthritis and other immune-mediated inflammatory diseases for the anti-inflammatory and immunomodulatory activity\(^10, 11\), is also very frequently used in therapy of cancer in Chinese hospitals. Some natural products derived from the herb, such as celastrol\(^12, 13\) and triptolide\(^14, 15\) were reported as potent anti-PCa agents. What is more, through a genomics-based phenotypic screening approach, triptophenolide was found as an effective inhibitor of AR\(^16\), however, the detailed anti-androgen mechanism remains unknown\(^17\). Herein, starting from the detection of the anti-androgen activity of ethanol extract of *Tripterygium wilfordii* root, we finally identified triptophenolide as a pan-antagonist for wild-type and mutant ARs, which inhibiting androgenic activity through competitive binding with androgen in the HBP of AR, decreasing the expression of androgen receptor, and reducing the nuclear translocation of AR.

The strategy was shown in Scheme1. We tried to screen the antiandrogen from *Tripterygium wilfordii* through combining MTT assay and dual-luciferase assay. As a start, we inspected the antiandrogenic potency of *Tripterygium wilfordii* root ethanol extract through MTT and dual-luciferase assay. In MTT assay, both LNCaP (AR positive) and PC-3 (AR negative) cell lines were used to evaluate the anti-prostate cancer activity of the herb. As a result, *Tripterygium wilfordii* root extract inhibited the growth of the LNCaP cells rather than the PC-3 cells in a significant dose-dependent manner at the concentration range of 1.0-7.5 µg/mL (FigS1 A). Such difference in cell growth inhibition between the LNCaP cells and the PC-3 cells of the extract suggested its inhibition of the AR activity, thus making the LNCaP cells more sensitive. Following dual-luciferase assay of AR activity in LNCaP cells confirmed that *Tripterygium wilfordii* root extract significantly inhibited the AR transcription activity at the concentration range of 5.0-7.5 µg/mL (FigS1 B), which elucidated the more sensitivity of LNCaP cells against the extract.

To inspect which components in *Tripterygium wilfordii* root should be responsible for the antiandrogen activity, we tested six main components from *Tripterygium wilfordii*, and the results were shown in FigS2. The luciferase assay of the AR activity in LNCaP cells revealed that all six components could significantly reduce the endogenous AR transcription activity (FigS2 A) at the concentration of 5 µM, and triptophenolide, triptolide, and triptonide displays significant anti-AR activity.
potency at the concentration of 500 nM. As AR mutant F876L
will cause the drug resistance of the clinical used antiandrogen
ENZ, we further investigated the antiandrogenic activities of
all these six components against the F876L mutant AR. AR-
dependent reporter assays were performed in PC-3 cells using
ARE (androgen receptor element) driven PSA-luc reporter and
AR-expressing plasmids. Antiandrogen ENZ was included as
the control. As a result, in the absence of dihydrotestosterone
(DHT), the F876L mutated AR was activated by ENZ (Fig S2B),
which was consistent to previous reports. Only tripto-
phenolide demonstrates the significant antiandrogenic activity
in suppressing DHT-induced transcription of the endogenous
F876L mutated AR at the concentration of 500 nM (Fig S2 B).
Thus we selected triptophenolide as a potential antiandrogen
for further mechanism studies.

To further evaluate the antiandrogenic potency of triptophe-
nolide, we investigated antiandrogenic activity of triptophe-
nolide against wild-type (WT) and T877A (FLU resistance
mutant), F876L, and W741C + T877A (FLU and BIC resis-
tance mutant) mutated ARs in absence or presence of DHT
at 50 nM, 500 nM, and 5 µM. As results, triptophenolide did
not show any agonistic activity to both the wild type and mu-
tated ARs in absence of DHT (Fig 1 A-D, white bars). In pres-
ence of DHT, triptophenolide demonstrates effective anti-
androgenic activity in suppressing DHT-induced transcriptions
of the WT, the F876L, T877A, and W741C+ T877A AR mu-
tants in a dose-dependent manner (Fig 1 A-D, black bars),
which indicates triptophenolide is a pan-antagonist against
WT and mutated ARs (The results of the luciferase assay of
the ARs activity for triptophenolide within the 0.1-25 µM con-
centration range were listed in Figure S3). We also evaluated
the IC_{50} value of triptophenolide against AR transcription ac-
activity. The result showed that triptophenolide has the IC_{50}
value of 260 nM for AR-WT, 480 nM for AR-F876L, 388 nM
for AR-T877A, and 437 nM for W741C+T877A (Figure S4),
respectively. Next, the regulation of PSA at RNA level by
triptophenolide was detected using RT-PCR in LNCaP cells.
The results showed that triptophenolide significantly down-
regulated PSA mRNA level at 500 nM and 5 µM (Fig 1E, the
measured value of RT-PCR assay were listed in Table S3). It
means that the inhibition of the PSA expression by triptophe-
nolide is modulated at mRNA level. Next, the in vitro cytotoxic-
ity of triptophenolide was evaluated by MTT assays in
LNCaP and PC-3 cells. As a result, triptophenolide inhibits the
growth of the LNCaP cells rather than the PC-3 cells in a sig-
ificant dose-dependent manner at the concentration range of
0.5-5.0 µM (Fig 1F). Such difference in cell growth inhibition
between the LNCaP cells and the PC-3 cells of triptophenolide
might be caused by its antiandrogenic activity.

To investigate whether triptophenolide exhibits its anti-
androgenic activity through competitive binding with andro-
gen in the HBP, the AR fluorescence polarization (FP) assay
was performed with PolarScreen AR competitor assay kit
(P3018, Invitrogen). When the tracer is free in solution, its
rotational mobility is greater than when bound to the receptor,
resulting in a low fluorescence polarization value. We have
controlled the assay for minimal competition (DMSO vehicle),
which has a maximum value of fluorescence polarization and
for no receptor (tracer only), which represents the minimum
value of the fluorescence polarization that can possibly be
reached by a competitor. The ENZ was included as a positive
control (Fig 2). Triptophenolide reduced the fluorescence po-
larisation value in a dose-dependent manner at the concentra-
tion range of 10 nM-10 µM, indicating it binds to AR-LBD by
competing with the tracer over the HBP (Fig 2A). The IC_{50}
value computed from the inhibition curve of triptophenolide
was 467 nM (Fig 2B), comparable to ENZ (294 nM, Fig 2B),
which indicated that triptophenolide most probably exhibits its
anti-androgenic activity via competitive binding with andro-
gen in the HBP.

Next, we predicted the binding mode of triptophenolide in
AR HBP through molecular docking using Molecular Operat-
ing Environment (MOE) version 2009.10. To date, crystal
structure of AR-LBD at the antagonist form has not been
obtained. Thus we built a structural model of the WT AR-
LBD with H12 at the antagonist form, using crystal struc-
tures of the AR-LBD/DHT and ER/antagonist complexes
(PDB entries: 1i65 and 3ert) as templates. Three other anti-
androgens (ENZ, BIC, FLU) were also docked as reference
compounds (Figure S5), and triptophenolide had the second
most favorable docked score after ENZ (Table S4). The pre-
dicted binding model of triptophenolide in the antagonist
model of WT AR-LBD was shown as Fig 2C. D. Triptophe-
nolide fits the HBP of AR quite well (Fig 2C), and it is as-
sumed to form hydrogen bond interactions with Gln711, 
Arg752, and Met780 as well as the hydrophobic interactions
with Leu701, Leu704, Leu707, Met742, Met745, Met749, 
Ph4764, Met780, Leu873 and Phe876 at the HBP (Fig 2D),
which indicates that triptophenolide should bind at the HBP in
a high specificity.

To determine whether triptophenolide would modulate AR
protein expression, we further examined the AR protein levels
during triptophenolide treatment. Western blot analysis indi-
cated triptophenolide effectively suppressed AR protein ex-
pression in LNCaP cells in a dose-dependent manner at 50
nM, 500 nM and 5 µM for 24 hours (Fig 3A). Thus, besides
competitive binding with androgen in HBP, triptophenolide is
also able to exert the antiandrogen potency through the down-
regulation of the AR protein level. Upon activated by andro-
gen, the AR translocates into the nucleus where it binds ARE
sites of DNA. Therefore, the confocal assay was done to eval-
uate the AR nuclear translocation in presence of triptophe-
nolide. As a result, in presence of 1nM DHT, the ratio of nu-
clear versus cytoplasmic AR in triptophenolide (5 µM) treated
cells was comparable to ENZ (500 nM) treated cell, threefold
reduced relative to DHT alone (Fig 3B). It indicates that trip-
ophenolide is able to inhibit DHT induced AR nuclear trans-
location and further confirms that triptophenolide is a bona
fide antiandrogen.

Here, we firstly discovered that the TCM herb Tripterygium
wilfordii could efficiently inhibit the AR transcription activity.
Further we identified triptophenolide from Tripterygium wil-
fordii as a pan-antagonist for wild-type and mutant ARs. Spe-
cially, triptophenolide has high binding affinity with AR, and
inhibits the activity of F876L mutant AR which turns ENZ
from AR antagonist to agonist. In addition, triptophenolide
could reduce the AR protein levels. Another resistance me-
chanism of ENZ during the treatment of PCa is the activation
of the glucocorticoid receptor (GR) signaling. It has been re-
ported that triptophenolide is also an inhibitor of GR
which could overcome the resistance caused by the bypassing
pathway activation of GR. Thus, triptophenolide may offer an
interesting anti-PCa lead compound for the further optimization.
Moreover, the work further supports that the TCM is able to
provide a potential resource of therapeutic agents for PCa, and
our strategy used here could also be extended to other TCM herbs to search for novel antiandrogens or other anti-PCa compounds.

Fig. 1. Triptophenolide is identified as an antiandrogen: A) Effect of triptophenolide on wild-type AR activity in PC-3 cells; B) Effect of triptophenolide on F876L AR activity in PC-3 cells. The F876L mutated AR was activated by ENZ at 500 nM; C) Effect of triptophenolide on T877A AR activity in PC-3 cells. The T877A mutated AR was activated by hydroxyflutamide at 5µM; D) Effect of triptophenolide on T877A+W741C AR activity in PC-3 cells. The T877A +W741C mutated AR was activated by both hydroxyflutamide and bicalutamide at 5µM; E) Triptophenolide significantly downregulated PSA mRNA level at 500nM and 5µM; F) Growth inhibitory effects of triptophenolide in the PC-3 and LNCaP cells. Plasmids expressing ARs are transiently transfected in PC-3 cells in dual luciferase assay. Experiments were in triplicate. *P < 0.05, **P < 0.01, ***P < 0.001 vs DMSO group, △P < 0.05, △△P < 0.01, △△△P < 0.001 vs DHT group. BIC: bicalutamide; ENZ: enzalutamide; DHT: dihydrotestosterone. All results are shown as mean ± s.d.

Fig. 2. A) Competitive binding of triptophenolide to the AR-LBD evaluated by AR fluorescence polarization (FP) assay; B) The inhibition curves of triptophenolide and ENZ; C) Triptophenolide (in stick, carbon in grey) fits the HBP of AR quit well in the predicted binding mode; D) The detail interactions between Triptophenolide and AR, hydrogen bond is indicated by the black dashed line with arrow. Experiments were in triplicate. *P < 0.05, ** P < 0.01,  *** P < 0.001 vs control group. All results are shown as mean ± s.d.

Fig. 3. A) Western blot analysis indicates that triptophenolide suppresses AR protein expression in LNCaP cells at 500nM and 5µM; B) Confocal assay indicates triptophenolide efficiently inhibit DHT induced AR nuclear translocation. Experiments were in triplicate. *P < 0.05, ** P < 0.01,  ***P < 0.001 vs control group. TRI: triptophenolide. All results are shown as mean ± s.d.

Scheme 1. The strategy in search of antiandrogen derived from *Tripterygium wilfordii.*
ASSOCIATED CONTENT

Supporting Information
NMR and MS analyses, biological assays details, and molecular modeling methods.
The Supporting Information is available free of charge on the ACS Publications website.

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Author Contributions
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Notes
Any additional relevant notes should be placed here.

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ABBREVIATIONS

AR, androgen receptor; ER, estrogen receptor; WT, wild type; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; PSA, prostate specific antigen; TCM, traditional Chinese medicine; LBD, ligand bind domain.

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Graphic Table

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