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Role of Breast cancer resistance protein (Bcrp/Abcg2) in Triptolide-induced Testis Toxicity

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Abstract

Triptolide have been intensively studied in numerous preclinical and clinical assessments for the immunosuppressive and anti-tumor activities. However, further clinical use is limited by the cumulative toxicity of triptolide in the testis and the mechanisms are poorly understood. In this study, we found significant triptolide accumulation in the testis, and further investigated the role of efflux transporters in the testis accumulation and toxicity of triptolide. Chronic administration of triptolide induced time- and dose-dependent testicular injury and resulted in the accumulation of triptolide in the liver and testis, but not in the plasma. Using transporter-expressed cells, triptolide efflux was found in BCRP-expressing cells, which could be blocked by novobiocin (an inhibitor of BCRP) in accumulation assays. Triptolide also displayed apically directed transport across BCRP-expressing cell layers in transwell assays, strongly supporting that triptolide is a substrate of BCRP. Bcrp knockout mice ($Bcrp^{-/-}$) were further used to examine the effects of triptolide. Knockout of Bcrp aggravated triptolide-induced testicular injury and increased the testis content and testis to plasma ratio of triptolide in $Bcrp^{-/-}$ mice. Notably, triptolide decreased the transcript and protein levels of Bcrp in the testis, which may be due to the downregulation of RNA polymerase II.

In conclusion, as a substrate of BCRP, triptolide decreased the expression of Bcrp and RNA polymerase II in the testis, and further increased testis content and enhanced its testicular toxicity, which contributes to the cumulative toxicity of triptolide in the testis.

Key Words: triptolide; breast cancer resistant protein; testicular injury

Introduction

Triptolide is a diterpene triepoxide purified from Tripterygium wilfordiiHook F (TWHF), a medicinal plant whose extracts have been used in traditional Chinese medicine for treating a wide variety of diseases from inflammation to arthritis for centuries.[1] It is structurally distinct in that it contains three epoxide groups next to each other (Fig. 1). It possesses a unique profile of biological activities including anti-inflammation, anti-proliferation and anti-cancer. Preclinical studies have revealed that triptolide has strong effects against cancer, collagen-induced arthritis, skin allograft rejection and bone marrow transplantation.[2-7] Several derivatives of triptolide have entered human clinical trials for cancer and other diseases.[8-11] However, the clinical uses of triptolide and its derivatives have been limited by their toxicity. Triptolide induced cumulative testis toxicity in some experimental and clinical researches.[9, 12-17] The mechanisms of testicular injury induced by triptolide are not well characterized.

It's well-known that drug transporters play a major role in drug distribution and drug sensitivity.[18-22] The best studied efflux transporters are multidrug resistance protein (MDR), multidrug resistance-associated protein (MRP), and breast cancer resistance protein (BCRP). In the testis, the blood-testis barrier is created by adjacent sertoli cells near the basement membrane and serves as a 'gatekeeper' to prohibit harmful substances from reaching the developing germ cells most notably post-meiotic spermatids. Recent studies have shown some efflux transporters expressed by spermatocytes, sertoli cells and spermatogonia including MDR1, BCRP, MRP1/2.[23-27]

Some studies have shown that efflux transporters (Mdr1 and Bcrp) expressed in the testis could limit the distribution of drugs and chemicals into the testis,[28, 29] but the roles of efflux transporters in triptolide-induced testicular injury and the resistance of sertoli cells have not been clarified. In this study, we found that triptolide accumulated in the testis. Accumulation and Transwell assay showed that triptolide was a substrate of BCRP. Bcrp limited the distribution of triptolide into the testis. Knocking out Bcrp increased triptolide distribution in the testis and caused more severe testicular injury.

Material and methods

Materials

Triptolide (99.9%) was kindly provided by Professor Yuanchao Li (Shanghai Institiute of Materia Medica, Shanghai, China). All other chemicals were commercially available and purchased as reagent grade from Sigma-Aldrich (St. Louis, USA).

Ethics Statement

Animal-use protocols were approved by the Institutional Animal Care and Use Committee of the Shanghai Institute of Materia Medica (Shanghai, China), IACUC No. 2014-07-RJ-89.

Animal care and study design

C57BL/6 mice, wild-type FVB mice and Bcrp knockout mice ($Bcrp^{-/-}$) FVB mice

(9-12weeks old) were used in this study. All animals were maintained under controlled temperature with a light/dark cycle. Food and water were provided ad libitum.

Toxicological study in C57BL/6 mice

Male C57BL/6 mice (9 weeks old, 22-24 g body weight) were used in studies of the oral administration and toxicity of triptolide (0.125 and 0.25mg/kg). Triptolide (0.125 and 0.25mg/kg; diluted with saline, DMSO content did not exceed 0.1% of the total volume) was consecutively administered for 15 days. The control group received saline containing 0.1% DMSO. Mice were sacrificed on the 3rd, 7th, 11th and 15th day post-administration and blood, liver, kidney, spleen, testis and epididymis were collected. The main lobe of liver, the kidney, the spleen and the epididymis were fixed in 10% neutral buffered formalin for histological examination, the left testicle was fixed in Davidson's buffer for 16 hours followed by 10% neutral buffered formalin. Tissue sections were stained with hematoxylin and eosin (H&E). Remaining tissues were stored at -80^{o}C until use.

Serum levels of blood urea nitrogen (BUN), creatine (CRE), alanine aminotransferase (ALT) and aspartate transaminase (AST) were determined using an automatic HITACHI Clinical Analyzer Model 7080 (Hitachi High-Technologies Corporation, Tokyo, Japan).

Tissue accumulation of triptolide in C57BL/6 mice

Twenty-four Male C57BL/6 mice (9 weeks old, 22-24 g body weight) were divided into four groups. Triptolide (0.5mg/kg) was consecutively administered. Mice were sacrificed 3h after final administration on the 3rd, 7th, 11th and 15th day and blood, liver, kidney, spleen, testis and epididymis were collected and stored at -80^{o}C until use.

Toxicokinetics study in wild type and Bcrp$^{-/-}$ FVB mice

Triptolide (0.25mg/kg) was dissolved in saline and orally administered for 11days. Mice were sacrificed 1 hour after final administration. Blood was collected from the left jugular vein and centrifuged at 4 ℃ and 1000 x g for 5 min to obtain plasma. After weighing, the liver, kidney, testis, epididymis and spleen were collected. PBS was added and tissues were then homogenized to yield a 20% homogenate for liver and kidney, and a 10% homogenate for testis, epididymis and spleen. All samples were stored at -80°C until use.

Pharmacokinetics study at steady state of i.v. infused triptolide in wild type and Bcrp$^{-/-}$ FVB mice

Under isoflurane anesthesia, the right jugular vein was cannulated with a polyethlene tube (PE-50; BD Biosciences, San Jose, CA). Triptolide was solubilized at a concentration of 3□g/mL in saline and continuously infused through the cannula at a dose rate of 200ng/min/kg. Blood was collected from the left jugular vein at 60, 90, 120, and 150 min and centrifuged at 4°C and 1000g for 5 min to obtain plasma. Immediately after the final blood sampling, mice were sacrificed by cervical

dislocation, and tissues were collected. PBS was added and tissues were then homogenized to yield a 20% homogenate for liver and kidney, and a 10% homogenate for testis, epididymis and spleen. All samples were stored at -80°C until use.

Toxicological study in wild type and Bcrp$^{-/-}$ FVB mice

Triptolide (0.25mg/kg) was dissolved in saline and orally administered for 11days. Mice were sacrificed 1 hour after final administration. After weighing, the liver, kidney, testis, epididymis and spleen were collected. All samples were stored at -80°C until use.

Cell culture, Accumulation and Transcellular transport assay

Mock, MDR1, BCRP and MRP2-MDCKII cells were used in the accumulation assays. Mock and BCRP-MDCKII cells were used in bidirectional transport studies. These cells were grown in a 5% CO_2 atmosphere at 37°C in DMEM culture medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1% antibiotic and antimycotic solution (Sigma Aldrich).

Mouse TM4 (sertoli cell-like, ATCC number: CRL-1715) and spermatocyte-like GC-2spd (ATCC number: CRL-2196) were purchased from ATCC (Manassas, VA, USA). TM4 was cultured in DMEM supplemented with 5% Horse serum and 2.5% fetal bovine serum. GC-2spd was cultured in DMEM supplemented with 10% fetal bovine serum. Cell viability was determined using the CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega, Madison, WI, USA).

Sample preparation and LC-MS/MS Analysis

Samples were precipitated with three (for in vitro samples) or four (for in vivo samples) volumes of ethyl acetate and centrifuged at 4°C and 15,000 x g for 10 min. After evaporation of the supernatants, the pellets were reconstituted with 70% mobile phase B (methanol)/30% mobile phase A (10mM NH_4Ac, 0.1% formic acid) and subjected to LC/MS/MS spectrometry analysis. An ABI SCIEX QTRAP 5500 equipped with a LC-20AD XR system (Shimadzu, Kyoto, Japan) was used for analysis. Analyst 1.5 software was used for data acquisition and processing. Samples were separated on an Atlantis dC18 column (5 □m, 2.1 x 50 mm; Waters, Ireland). Triptolide was eluted with a mobile phase of methanol-water containing 0.1% formic acid and 10mM NH_4Ac (70:30, v/v) at a flow rate of 200□L/min. The retention time of triptolide is 1.68min. Conditions for MS analysis of triptolide included an ion spray voltage of 5500 V and a temperature of 300°C. Collision and curtain gas flow were 4 and 30 mL/min, respectively. The fragment of triptolide was induced with collision energy of 15 eV. The optimized declustering potential, entrance potential, and collision cell exit potential were 66, 10 and 11 V, respectively. MS was performed in a positive mode under multiple reaction monitoring. The m/z ratio of the parent and product ions of triptolide was 378.2(+NH4) and 361.2(+H). The lower limit of quantification for triptolide was 0.2ng/ml. The calibration curve was linear over the concentration range (0.2 to 1000 ng/ml).

Quantitative real-time PCR (qPCR)

Total RNA from mouse testis was extracted using the UNIQ-10 total RNA isolation kit (Sangon Biotech, Shanghai, China). Remaining genomic DNA in the total RNA was digested using RNase-free DNase I (Fermentas, Burlington, Canada). DNase-digested RNA was reverse transcribed into cDNA using the PrimeScript RT reagent kit (TaKaRa, Otus, Shiga, Japan). DNase-digested RNA without reverse transcription was used as a negative control. qPCR was carried out using the SYBR Premix Ex Taq (TaKaRa) with primers for GATA binding protein 1 (gata1, NM_008089) (forward: 5-CAGGTTTCTTTTCCTCTGGG-3, reverse: 5-AAAGGACTGGGAAAGTCAGC-3), phosphoglycerate kinase-2 (pgk2, NM_031190) (5-CTGTTGCTGATGAGCTCAA G-3, reverse: 5-ACTCCGACCATAGAACTGTG-3), Zbtb16 zinc finger and BTB domain containing 16 (Plzf, NM_001033324)(Forward: 5-TGAGATCCTCTTCC ACCGAA-3, Reverse: 5-GTAGGACTCATGGCTGAGAGA-3), Deleted in azoospermia-like (Dazl, NM_010021)(Forward: 5-CCTCCAACCATGATGAAT CC-3, Reverse: 5-TCTGTATGCTTCGGTCCACA-3), Protamine 1(Prm1, NM_013637)(Forward: 5-ATGCTGCCGCAGCAAAAGCA-3, Reverse: 5-CACCTTATGGTGTATGAGCG-3), Breast cancer resistance protein (Bcrp, NM_011920) (forward: 5-GAACTCCAGAGCCGTTAGGAC-3, reverse: 5-CAGAATAGCATTAAGGCCAGGTT-3)and gapdh (NM_008084) (forward: 5-GGCTACACTGAGGACCAGGTT-3, reverse: 5-TGCTGTAGCCGTATTCATTGT C-3). qPCR cycle parameters were: 95^{o}C for 10 s, followed by 40 cycles of 95^{o}C for 5s, 60^{o}C for 30s) with a melting-curve analysis at the end of the protocol. The amplification process was performed on a 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) and data was analyzed by $2^{-\Delta\Delta Ct}$ method using the Sequence Detection Software.

Western blotting

Standard western blotting analysis was performed using monoclonal antibodies against hspa2 (Abnova, Taiwan, China), BCRP (BXP53), RNA polymerase II (Abcam, Cambridge, UK) and β-actin (Santa Cruz, California, USA).

Statistical Analysis

Statistical analysis was performed using one-way ANOVA or two-tailed Student's t tests. A probability of < 0.05 was considered to be statistically significant.

Results

Triptolide dose-dependently caused cell-selective testis injury and accumulated in the testis of mice

In the present study, triptolide (0.125-0.25mg/kg) reduced the testis weight of mice while having no obvious toxic effects on the liver, kidney, spleen and epididymis (Fig. 2B & C and Fig. S1 in supplementary material). Blood routine examination also did not find any abnormality in the circulatory systems (data not shown). Histological

analysis of testis revealed that the loss of testis weight may be due to the loss of spermatocytes (arrow head) and spermatids (arrow) (Fig. 2D-F).

Triptolide is rapidly eliminated with a 20min half-life in vivo.[30, 31] In this study, 3h after final administration, the tissue content of triptolide was detected to indicate the accumulation of triptolide, and 0.5mg/kg triptolide was used to make the testis content of triptolide to be higher than the detection limit of triptolide (0.2ng/mL). As Fig. 2G-I shown, in the first three days, triptolide could not be detected in the liver, testis and plasma. After 7days administration, triptolide was detected in the testis of two mice. As triptolide administration went on, the number of mice with detectable triptolide and the tissue content of triptolide in the liver and testis increased (Fig. 2G-H), strongly indicating the accumulation of triptolide in the liver and testis, but not in plasma.

Spermatogenesis is the process in which spermatozoa are produced from germ stem cells by way of mitosis and meiosis. To further elucidate mechanisms underlying the triptolide-induced testis toxicity, we investigated transcript levels of germ cell-specific differentiation markers.[32] We found that Gata1 (a marker for sertoli cells) and Plzf (a marker for germ stem cell and spermatogonial differentiation) were normally expressed in testis (Fig. 3A-B),[33] while Dazl (a marker for Type B spermatogonia and primary spermatocytes),[34, 35] Pgk2 (a meiotic spermatocyte marker) and Hspa2 (a marker for post-meiotic spermatocytes and spermatids) were decreased from 3th or 15st day (Fig. 3C-E).[36-38] Prm1 (a spermatids-specific marker) was rapidly decreased from 7th day (Fig. 3F).[39] Using spermatocyte-like cell line GC-2spd and sertoli like cell line TM4, we also found that GC-2spd was more sensitive to triptolide than TM4 (Table 1). These data indicated that sertoli cells were more resistant to triptolide than other germ cells, suggesting a potential efflux of triptolide by ABC transporters expressed in sertoli cells as the main component of the blood-testis barrier.

Triptolide is a substrate of BCRP

Some studies have shown the expression of efflux transporters in spermatocytes, sertoli cells and spermatogonia including MDR1, BCRP, MRP1/2.[23-27] We used MDR1, BCRP and MRP2 expressed MDCKII cell line to examine the effect of each gene on triptolide distribution.

In intracellular accumulation assays, triptolide efflux was only observed in BCRP-MDCKII cells and triptolide could not be fluxed by MDR1 or MRP2-MDCKII cells (Fig. 4 and Fig. S2 in supplementary material). Novobiocin (100μM) was used as a BCRP inhibitor and effectively inhibited triptolide efflux at different time points (Fig. 4A) and under different triptolide concentrations (Fig. 4B).

The transport of triptolide was further investigated using BCRP MDCKII cells. BCRP is localized in the apical membrane. In BCRP MDCKII cells, the permeability of basal-to-apical direction was greater than that of apical-to-basal direction for triptolide, whereas the transcellular transport was almost identical in both directions in mock-transfected MDCK II cells (Fig. 4C). ^{3}H prazosin, a substrate of BCRP, was used as a positive control (Fig. 4D). Collectively, these results suggested that triptolide is a substrate of BCRP.

Bcrp knockout increased testis content and aggravated testis toxicity of triptolide

Triptolide (0.25mg/kg) was dissolved in saline and orally administered for 11days. Mice were sacrificed 1 hour after final administration. Tissue distribution of triptolide was compared between WT and Bcrp$^{-/-}$ mice. Triptolide content was much higher in the testis of Bcrp-/- mice than that of WT mice (2.55 fold that of WT mice). The testis to plasma ratio was also higher in Bcrp$^{-/-}$ mice than in WT mice (1.76 fold that of WT mice) (Fig. 5A, B).

The tissue distribution of triptolide in WT and Bcrp$^{-/-}$ mice was further examined at steady state by utilizing continuous intravenous infusion. The plasma concentration of triptolide remained nearly constant between 60 to 150 min, indicating that the plasma concentration reached a plateau at 60 min. No significant difference in the plasma concentration of triptolide was observed between WT and Bcrp$^{-/-}$ mice (data not shown). However, the triptolide content (1.74-fold that of WT mice) and the testis to plasma ratio (1.6-fold that of WT mice) of testis were significantly increased in Bcrp$^{-/-}$ mice (Fig. 5C, D). The testis to plasma ratio of the epididymis was also significantly increased in Bcrp$^{-/-}$ mice by 1.6-fold over WT mice (Fig. 5D).

After 11-day administration of triptolide (0.25mg/kg, orally) Bcrp$^{-/-}$ mice had lower testis weight, indicating more severe testis toxicity (Fig. 5E, F).

Triptolide decreased the expression of Bcrp and RNA pol II

We further detected the effects of triptolide on the expression of Bcrp. The transcript and protein levels of Bcrp were obviously decreased by triptolide in the testis of mice and sertoli-like TM4 cells (Fig. 6A-C). As a potential target of triptolide,[7] RNA polymerase II in TM4 and GC-2spd cells was also downregulated by triptolide (Fig. 6C) after 16h exposure.

Discussion

Triptolide could cause damage in several tissues including the digestive, urogenital, circulatory and reproductive systems.[14, 15] In this study, triptolide specifically damaged testis (Fig. 2, 3, S1; Table 1). In vitro screening with drug transporters expressing cell lines found that triptolide was a substrate of BCRP (Fig. 4, S2). Knocking out bcrp increased the severity of triptolide-induced testis toxicity, which may be due to the higher triptolide content in the testis of Bcrp$^{-/-}$ mice (Fig. 5). It's worth noting that triptolide decreased the expression of Bcrp *in vivo* and *in vitro* (Fig. 6). Together, our data suggested that, as a substrate of Bcrp, triptolide induces cumulative testis toxicity through decreasing the expression of Bcrp in the testis.

In our study, knockout and pharmacological inhibition of Bcrp moderately increase the toxicity of triptolide (Fig. 5, Table 1), which may be due to the low affinity of triptolide for BCRP (K_m = 661.3 ± 117.3 □M). On the other hand, TM4 showed high resistance to triptolide comparing to GC-2spd cells (Table 1). Other factors like heat shock protein a2 (Hspa2, formerly hsp70.2) may be involved in the great difference in cytotoxicity between TM4 and GC-2spd. Hspa2 is a spermatocyte-specific member of the hsp70 family that is known to play a critical role in meiosis,[40] and some studies have reported that hsp70 and its transcriptional regulator (heat shock factor 1, Hsf1) are specific targets of triptolide.[5, 41] Here, we also

found an obvious decrease of hspa2 protein level induced by triptolide (Fig. 3E). More works are needed for the role of hspa2 or Hsf1 in triptolide-induced testis injury.

BCRP is expressed at the apical membrane of hepatocytes and epithelial cells of the intestines and kidney where it pumps a wide variety of endogenous and exogenous compounds out of the cell.[42] We examined the effect of a Bcrp inhibitor, novobiocin, on the absorption of triptolide in vivo. Oral co-administration of novobiocin increased the plasma concentration of triptolide in male FVB mice. The area under curve over 2h ($AUC_{0\text{-}120min}$) of co-administration was 1.55-fold that of single administration of triptolide (Fig. S3). Novobiocin decreased the oral clearance of triptolide (from 0.34±0.01L/min/Kg to 0.17±0.04 L/min/Kg) and increased the exposure to triptolide. These co-administration results demonstrated a potential drug-drug interaction between triptolide and inhibitors of BCRP.

Two studies from Swerdloff and colleagues in rats have shown that 0.1mg/kg triptolide (treatment for 70-82 days) only induced subtle testis change in SD rats, but greatly decreased cauda epididymal sperm content.[43, 44] In this study, Consecutive administration of triptolide (0.0625mg/kg-0.125mg/kg) for 60 days caused severe testis injury in C57BL/6 mice (Fig. S4). Triptolide is extensively metabolized in SD rats with a half-life about 20min[30]. However, in C57BL/6 mice and FVB mice, the half-life is about 30min (Fig. S3), indicating stronger ability of SD rats to eliminate triptolide, which may contribute to the difference between SD rats and mice. Some other mechanisms should be involved in this difference and more work is needed.

In our preliminary experiments, triptolide (0.0625-0.5mg/kg) was consecutively administered for 60 days (Fig. S4). The mice in the 0.125-0.5mg/kg groups were infertile (data not shown). Bcrp knockout mice are fertile with normal feeding condition. We did not investigate the fertility of Bcrp knockout mice treated with triptolide. However, based on the results from Fig. S4, we suggested that if Bcrp knockout mice were continuously administered with triptolide (0.25mg/kg) for 60 days, they would lose their fertility.

The expression and distribution of Bcrp in the testis has been extensively studied and shows obvious species specificity. Some excellent researches in the rat testis performed by C. Yan Cheng's team, showed that Bcrp is mainly expressed in peritubular myoid cells and spatiotemporally expressed by sertoli cells and step 19 spermatids.[45-47] Several studies with FVB mice showed that Bcrp is positive in the luminal membrane of endothelial cells.[28, 48] While in the testis of C57BL/6 mice, elongated spermatids show strong immunoreactivity at the luminal compartment. Some positive cells are also observed in the adluminal area close to the basement, the localization for spermatogonia. The following RT-PCR further shows that Bcrp mRNA can be detected in germ stem cells, type A spermatogonia, spermatocyte I, spermatocyte II and spermatids (Figure 1 and 2 in Ref. 49).[49] In this study, triptolide caused the loss of spermatocytes and spermatids (Fig. 2-3). The mRNA and protein levels of Bcrp were both reduced by triptolide in vivo and Bcrp protein was also decreased by triptolide in TM4 cells (Fig. 6). Taken together, our results suggested that both the loss of spermatocytes/spermatids and Bcrp reduction by triptolide in

sertoli cells may contribute to the dramatic loss of Bcrp in the testis.

In this study, the C_{tissue}/C_{plasma} (Kp value) was determined under steady state conditions. These values represent the ratio of the clearance of uptake transport to that of efflux transport.[29] Here, the plasma concentration of triptolide was mildly higher in $Bcrp^{-/-}$ mice (Fig. S5). However, the testis and epididymis concentration of triptolide was increased significantly in $Bcrp^{-/-}$ mice, indicating Bcrp may affect the efflux transport of triptolide in the testis.

Outside the haematopoietic system, Gata1 is only expressed in the sertoli cells of testis. A sertoli cell-specific knockout of Gata1 reveals that gata1 is not essential for sertoli cell function.[50] Its profile follows the percentage of Sertoli cells in the testis.[51-53] After treatment with triptolide, Gata1 appeared to be upregulated at day 15, which coincides with the absence of pachytene spermatocytes and early spermatids (Fig. 3). This suggested that the apparent up-regulation likely was caused by changes in cellularity and that the level of Gata1 in sertoli cells probably was not affected by triptolide but remained constant.

BCRP is also important for maintaining the pharmacological sanctuary properties of several tissues, due to its expression in the blood-brain, blood-placental and blood-testis barriers.[42] There are two types of SNP variants of BCRP that are associated with reduced expression, the allele frequency of one of the variants (Q141K) in Japanese subjects is as high as 29-36%.[54] In the present study, we found that triptolide was a substrate of BCRP, which should prompt clinical attention to the patients with BCRP SNPs when they were treated with TWHF or triptolide.

In summary, we confirm that testis is the most sensitive target for triptolide and firstly find that triptolide is a substrate of BCRP. Bcrp influences the distribution and toxicity of triptolide in testis and partially mediated the resistance of sertoli cells. Our findings will contribute to the clinical application and derivatives development of triptolide.

Conflict of interest

All authors disclose any actual or potential conflict of interest.

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Figure legends

Fig. 1. **Structure of triptolide.**

Fig. 2. **Triptolide dose-dependently caused cell-selective testis injury and**

accumulated in the testis of C57BL/6 mice. (A) Administration of triptolide (0.125, 0.25mg/kg) for 15days did not affect the body weight of mice. (B) Testis weight of C57BL/6 mice. * $p<0.05$ vs Saline group, *** $p<0.001$ vs Saline group. (C) Relative testis weight of C57/B6L mice. Testis weight was normalized by body weight of the mice. * $p<0.05$ vs Saline group, *** $p<0.001$ vs Saline group. (D-F) Normal and damaged seminiferous tubule in the testis of C57BL/6 mice (H&E-stained sections, 40X). Arrow heads indicate spermatocytes, arrows indicate spermatids. Loss of spermatocytes and spermatids was found in (F). (G-I) Triptolide content in the liver, testis and plasma. The tissue content of triptolide was detected 3h after final administration at the indicted time points. 0.5mg/kg triptolide was administered. TL: triptolide. All the data are presented as the mean ± S.D.; n = 6.

Fig. 3. **Cell-selective testis injury induced by triptolide after 15-day treatment in C57BL/6 mice.** The mRNA levels of germ cell-specific differentiation markers in the testis were measured by real-time quantitative PCR. Triptolide was administered for 15 days. (A) mRNA level of gata1 in testis. Gata1 is a marker of sertoli cells; (B) mRNA level of plzf in the testis. Plzf is a marker for germ stem cells and spermatogonia differentiation; (C) mRNA level of dazl in the testis. Dazl is a marker for spermatognia; (D) mRNA level of pgk2 in testis. Pgk2 is a a meiotic spermatocyte marker; (E) Protein level of hspa2 in the testis after 15-day treatment of triptolide. Hspa2 is a marker for post-meiotic spermatocytes and spermatids, and was measured by western blotting; (F) mRNA level of prm1 in the testis. Prm1 is a marker for spermatids. TL: triptolide. All the mRNA levels of germ cell markers are normalized to gapdh. * $p<0.05$ vs Saline group, ** $p < 0.01$ vs Saline group, *** $p < 0.001$ vs Saline group. All the data are presented as the mean ± S.D.; n = 6.

Fig. 4. **Triptolide is a substrate of BCRP.** (A-B) Triptolide efflux by BCRP MDCKII cells in accumulation assay. (A) Mock and BCRP MDCKII cells were incubated with triptolide (5□M) in presence or absence of novobiocin (100□M) for 0.5, 1.5, 3, 6 and 15 min. (B) Mock and BCRP MDCKII cells were incubated with triptolide (0.5, 5, 20 and 50□M) in presence or absence of novobiocin (100□M) for 15 min. (C-D) Transport of triptolide by BCRP-MDCKII cells. The transcellular transport of triptolide(5□M) (C), prazosin (D) across monolayers of Mock and BCRP MDCK II cells. All the data are presented as the mean ± S.D.; n = 4.

Fig. 5. **Bcrp knockout increased testis content and aggravated testis toxicity of triptolide**. (A-B) Comparison of tissue distribution of triptolide between wild-type and $Bcrp^{-/-}$ mice at single dose. Triptolide (0.25mg/kg) was orally administered for 11days. Mice were sacrificed 1 hour after final administration. Bcrp knockout increased the distribution of triptolide in testis. All the data are presented as the mean ± S.D.; n =4. (C-D) Comparison of tissue distribution of triptolide between wild-type and $Bcrp^{-/-}$ mice at steady state. Triptolide was infused intravenously at a dose rate of 200ng/min/kg and tissue-to plasma values were measured at 150 min. Bcrp knockout increased the distribution of triptolide in testis. All the data are presented as the mean

± S.D.; n=3. (E-F) Triptolide (0.25mg/kg) was orally administered for 11days. Mice were sacrificed 1 hour after final administration. Testis weight (E) and relative testis weight (F) of WT and Bcrp$^{-/-}$ FVB mice were weighed. Triptolide caused significant decrease of testis weight in Bcrp$^{-/-}$ FVB mice on the 11th day.WT: wild type, Bcrp$^{-/-}$: Bcrp knockout. All the data are presented as the mean ± S.D.; n =4.

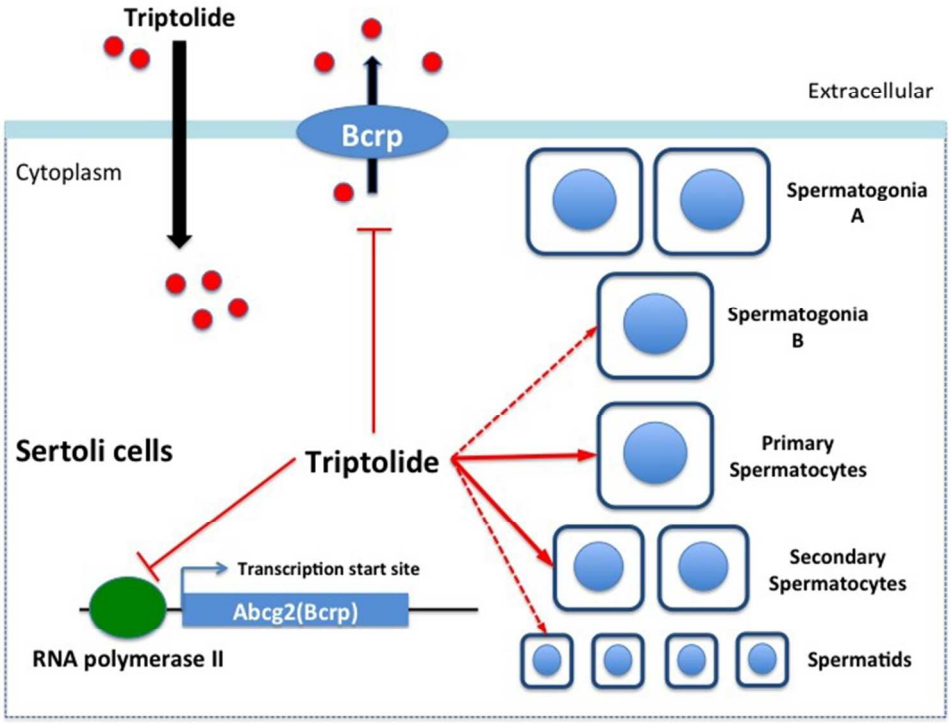
Fig. 6. **Triptolide decreased the expression of Bcrp and RNA pol II.**
Triptolide (0.0625-0.25mg/kg) was administered for 15days in C57/BL6 mice. The mRNA level (A) and protein level (B) of Bcrp in the testis were measured by real-time quantitative PCR and western blotting, respectively. (C) After 16h exposure with triptolide, the protein levels of Bcrp and RNA polymerase II (RNA pol II) in TM4 and GC-2spd cells were measured by western blotting. Data are presented as the mean ± S.D.; n =4. * $p<0.05$ vs saline group; ** $p<0.01$ vs saline group.

Table 1. IC_{50} of triptolide in the testis-derived cell lines.

Cell lines	**IC_{50} (nM) for triptolide**

TM4	5147 ± 404
TM4 (NOV)*	3224 ± 325
GC-2spd	72.4 ± 10.2

* NOV: novobiocin (25□M), an inhibitor of Bcrp, was co-incubated with triptolide. TM4: sertoli-like cell line, GC-2spd: spermatocytes-like cell line.



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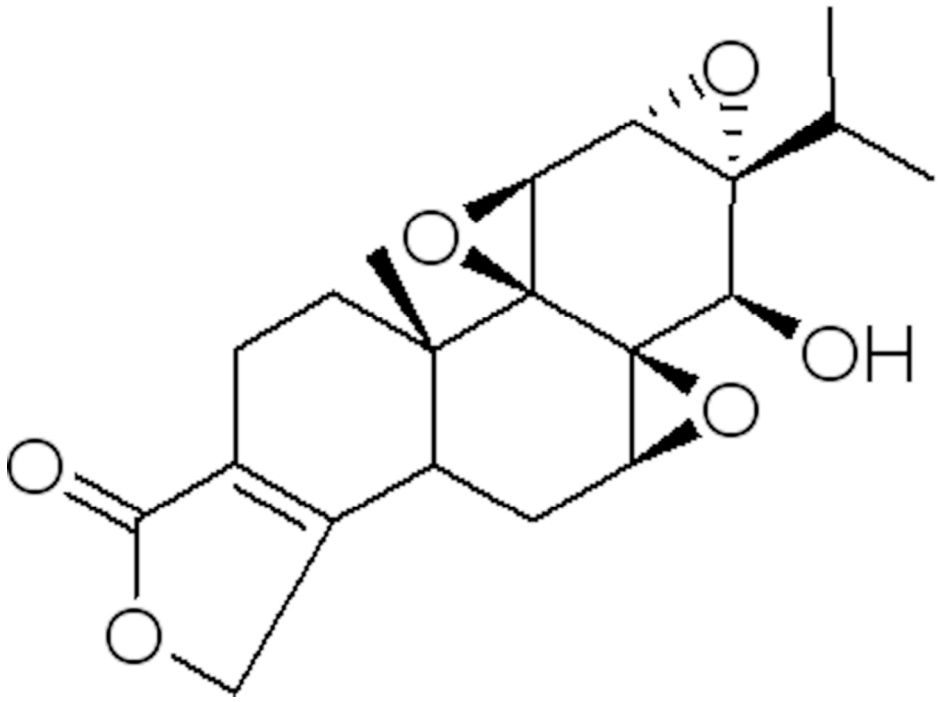

Structure of triptolide.
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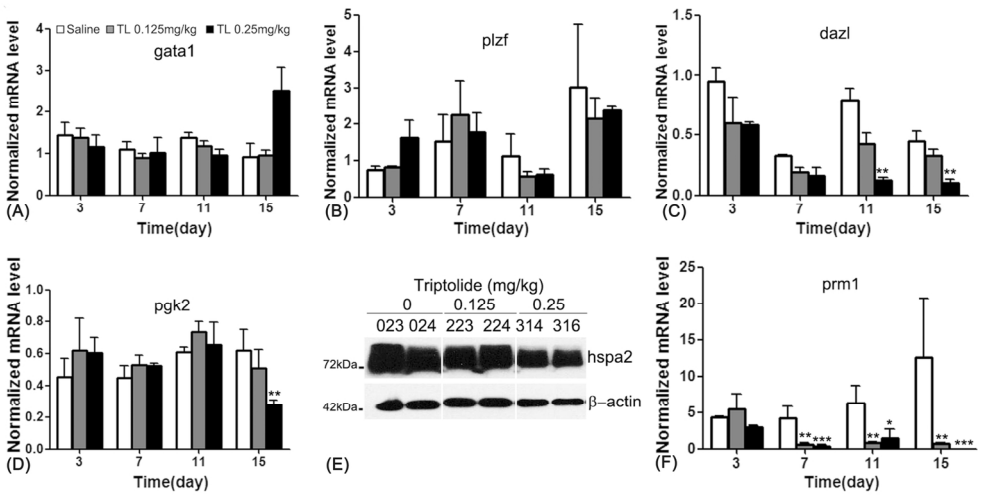

Cell-selective testis injury induced by triptolide after 15-day treatment in C57BL/6 mice. The mRNA levels of germ cell-specific differentiation markers in the testis were measured by real-time quantitative PCR. Triptolide was administered for 15 days. (A) mRNA level of gata1 in testis. Gata1 is a marker of sertoli cells; (B) mRNA level of plzf in the testis. Plzf is a marker for germ stem cells and spermatogonia differentiation; (C) mRNA level of dazl in the testis. Dazl is a marker for spermatognia; (D) mRNA level of pgk2 in testis. Pgk2 is a a meiotic spermatocyte marker; (E) Protein level of hspa2 in the testis after 15-day treatment of triptolide. Hspa2 is a marker for post-meiotic spermatocytes and spermatids, and was measured by western blotting; (F) mRNA level of prm1 in the testis. Prm1 is a marker for spermatids. TL: triptolide. All the mRNA levels of germ cell markers are normalized to gapdh. * $p<0.05$ vs Saline group, ** $p < 0.01$ vs Saline group, *** $p < 0.001$ vs Saline group. All the data are presented as the mean ± S.D.; n = 6.
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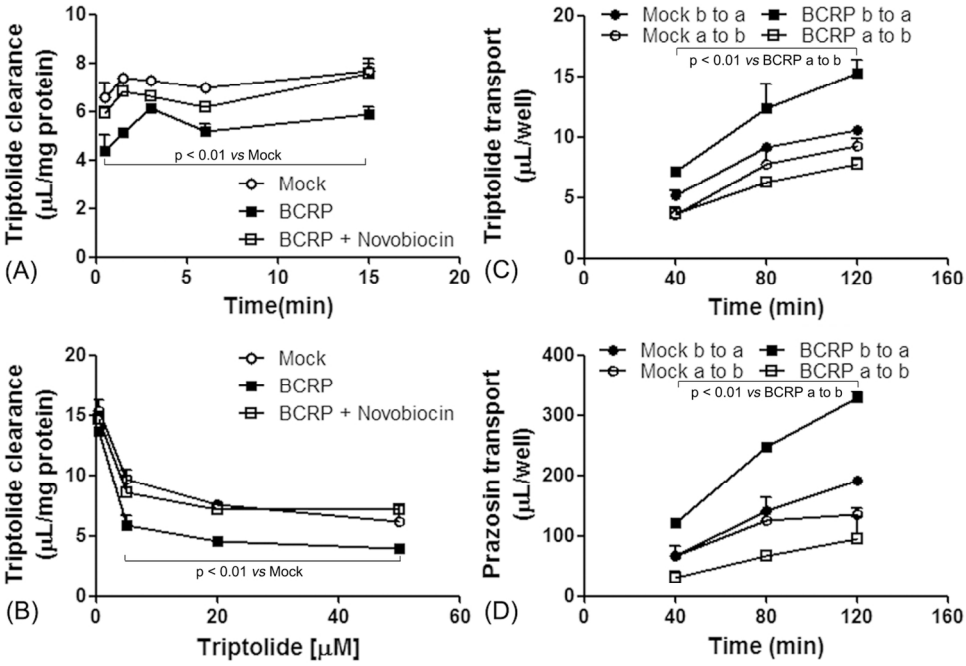

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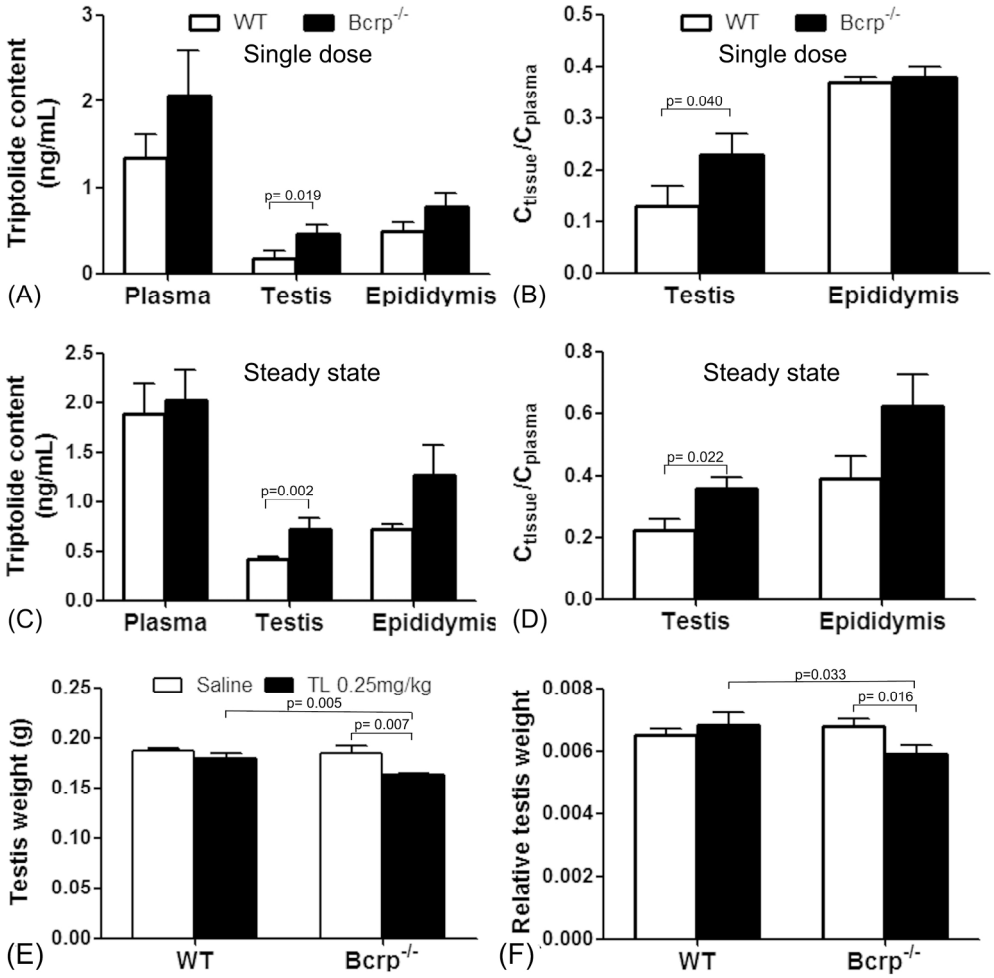

Bcrp knockout increased testis content and aggravated testis toxicity of triptolide. (A-B) Comparison of tissue distribution of triptolide between wild-type and Bcrp-/- mice at single dose. Triptolide (0.25mg/kg) was orally administered for 11days. Mice were sacrificed 1 hour after final administration. Bcrp knockout increased the distribution of triptolide in testis. All the data are presented as the mean ± S.D.; n =4. (C-D) Comparison of tissue distribution of triptolide between wild-type and Bcrp-/- mice at steady state. Triptolide was infused intravenously at a dose rate of 200ng/min/kg and tissue-to plasma values were measured at 150 min. Bcrp knockout increased the distribution of triptolide in testis. All the data are presented as the mean ± S.D.; n=3. (E-F) Triptolide (0.25mg/kg) was orally administered for 11days. Mice were sacrificed 1 hour after final administration. Testis weight (E) and relative testis weight (F) of WT and Bcrp-/- FVB mice were weighed. Triptolide caused significant decrease of testis weight in Bcrp-/- FVB mice on the 11th day.WT: wild type, Bcrp-/-: Bcrp knockout. All the data are presented as the mean ± S.D.; n =4.
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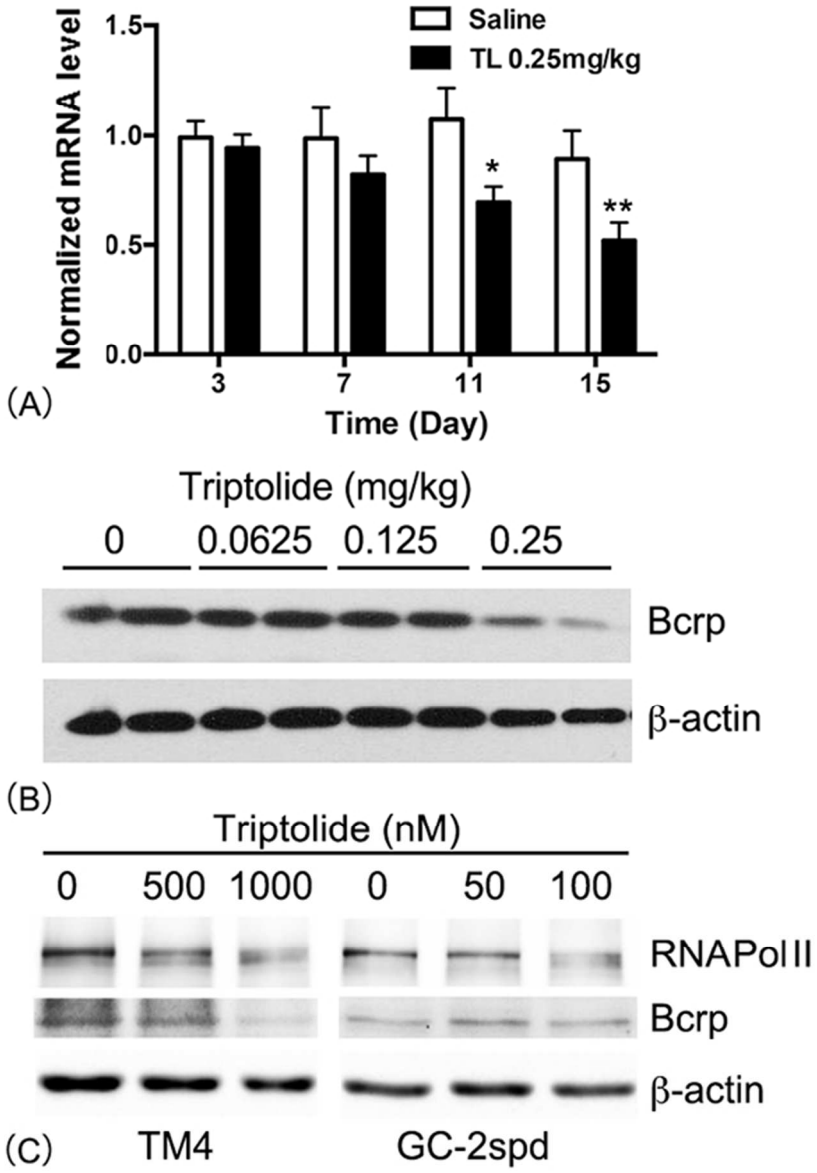

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