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The (+)- and (−)-gossypols potently inhibit both 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase 3 in human and rat testes☆

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ABSTRACT

Androgen deprivation is commonly used in the treatment of metastatic prostate cancer. The (−)-gossypol enantiomer has been demonstrated as an effective inhibitor of Bcl-2 in the treatment of prostate cancer. However, the mechanism of gossypol as an inhibitor of androgen biosynthesis is not clear. The present study compared (+)- and (−)-gossypols in the inhibition of 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-HSD isoform 3 (17β-HSD3) in human and rat testes. Gossypol enantiomers were more potent inhibitors of rat 3β-HSD with IC_{50}s of ~0.2 μM compared to 3–5 μM in human testes. However, human 17β-HSD3 was more sensitive to inhibition by gossypol enantiomers, with IC_{50}s of 0.36 ± 0.09 and 1.13 ± 0.12 for (−)- and (+)-gossypols, respectively, compared to 3.43 ± 0.46 and 10.93 ± 2.27 in rat testes. There were species- and enantiomer-specific differences in the sensitivity of the inhibition of 17β-HSD3. Gossypol enantiomers competitively inhibited both 3β-HSD and 17β-HSD3 by competing for the cofactor binding sites of these enzymes. Gossypol enantiomers, fed orally to rats (20 mg/kg), inhibited 3β-HSD but not 17β-HSD3. This finding was consistent with the *in vitro* data, in which rat 3β-HSD was more sensitive to gossypol inhibition than rat 17β-HSD3. As the reverse was true for the human enzymes, gossypol might be useful for treating metastatic prostate cancer.

1. Introduction

Androgen deprivation therapy is the main treatment for metastatic prostate cancer. However, prostate cancer can recur in an androgen-independent form in these patients. The anti-apoptotic role of Bcl-2 in androgen-independent prostate cancer cells has been proposed [1]. Resistance to androgen deprivation therapy may be associated with the enhanced expression of apoptotic proteins such as the Bcl-2 family of anti-apoptotic protein regulators. This family is composed of at least 16 members including anti-apoptotic proteins (Bcl-2, Mcl-1, Bcl-x_L) and pro-apoptotic proteins (Bax, Bak, and Bad). The balance of anti- and pro-apoptotic proteins determines the sensitivity or resistance of cells to apoptotic stimuli. Enhanced Bcl-2 expression has been demonstrated in prostate cancer cells when they become androgen-independent after *in vivo* castration to deplete androgen levels [2]. These observations have led to the proposal of the Bcl-2 family proteins as a therapeutic target for prostate cancer.

Gossypol is a yellowish polyphenolic compound isolated from cottonseeds that was once used as a male contraceptive in China [3]. Gossypol naturally occurs as enantiomeric (+)-gossypol and (−)-gossypol mixture. Only (−)-gossypol has antifertility action [4][5]. Thus (+)-gossypol had been dismissed as lacking potential clinical application. Recently, (−)-gossypol, not (+)-gossypol was shown to inhibit Bcl-2 by acting as a BH3 mimetic and disrupting the hetero-dimerization of Bcl-2 with pro-apoptotic family members. (−)-Gossypol mimics the pro-apoptotic proteins by binding

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to the BH3 domains of Bcl-2, Mcl-1, and Bcl-x_L [6]. (−)-Gossypol is therapeutically active in several cancer models including prostate, breast, colon and non-small cell lung cancer [7–10]. For prostate cancer therapy, (−)-gossypol also delays the onset of androgen-independent prostate cancer *in vivo* in combination with castration [11].

Gossypol inhibits steroidogenesis in bovine luteal cells [7] and may have clinical application in the inhibition of steroid synthesis. In the present study, we compared the potencies of both (+)- and (−)-gossypols as inhibitors of 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase 3 (17β-HSD3) in human and rat testes.

2. Materials and methods

2.1. Chemicals and animals

[1β,2β-^{3}H]Androstenedione (41.8 Ci/mmol), [1,2,6,7-^{3}H]pregnenolone (110 Ci/mmol) were purchased from Dupont-New England Nuclear (Boston, MA). Cold androstenedione, pregnenolone, progesterone and testosterone were purchased from Steraloids (Wilton, NH). (+)- and (−)-Gossypols were gifts from Dr. Samuel S. Koide at The Population Council. Male Sprague–Dawley rats (250–300 g) were purchased from Charles River Laboratories (Wilmington, MA). The experimental protocol was approved by the Rockefeller University's Animal Care and Use Committee (Protocol #07080). Human male testes were obtained from National Disease Research Interchange (Philadelphia, PA).

2.2. Preparation of microsomal protein

Microsomal preparations of human and rat testes were prepared as described previously [12]. In brief, testes were homogenized in cold 0.01 M PBS buffer containing 0.25 M sucrose and centrifuged at 700 × *g* for 30 min. The supernatants were transferred to new tubes and centrifuged at 10,000 × *g* for 30 min. The supernatants were centrifuged twice at 105,000 × *g* for 1 h (twice). Pellets were resuspended and protein contents were measured. The protein concentrations were measured by Bio-Rad Protein Assay Kit (cat #500-0006, Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Microsomes were used for measurement of 3β-HSD and 17β-HSD3 activity.

2.3. 3β-HSD assay

3β-HSD activity was measured in glass tubes that contained 200 nM pregnenolone and 40,000 dpm ^{3}H-pregnenolone in 0.1 mM PBS buffer. The substrate concentration was selected based on the interstitial fluid testosterone levels [13]. The 30 min reactions were initiated by addition of 30 μg human and 6 μg rat testis microsome proteins with 0.2 mM NAD^+ in presence of different concentrations of (+)- or (−)-gossypol to determine Half-maximal inhibitory concentration (IC_{50}). The reactions were performed at 34 °C in a shaking water bath (75 rpm). The incubation time was preliminarily determined to be within linearity of 3β-HSD activity.

To determine the inhibitory mode of gossypol enantiomers, different concentrations of pregnenolone (0.002–10 μM) plus 0.2 mM NAD^+ were added into the reaction mixture (PBS buffer, pH 7.2) containing 6 μg rat testis microsome and each gossypol enantiomer (1 or 10 μM). To determine the inhibitory mode of gossypol enantiomers via competition with NAD^+, different concentrations of NAD^+ (0.002–10 μM) plus 200 nM pregnenolone were added into the PBS reaction mixture containing 6 μg rat testis microsome and each gossypol enantiomer (1 or 10 μM). A preliminary experiment had determined the velocity of 3β-HSD within the linear range in the above conditions. The reactions were stopped by adding 2 ml ice-cold ether. The steroids were extracted and the organic layer was dried under nitrogen. The steroids were separated chromatographically on thin-layer plates in chloroform and methanol (97:3, v/v), and the radioactivity was measured using a scanning radiometer (System AR2000, Bioscan Inc., Washington, DC). The percentage conversion of pregnenolone to progesterone was calculated by dividing the radioactive counts identified as progesterone by the total counts associated with pregnenolone plus progesterone.

2.4. 17β-HSD3 assay

17β-HSD3 activity was measured in a similar manner to the above conditions with the following exceptions: the substrate of 200 nM androstenedione and 40,000 dpm ^{3}H-androstenedione, cofactor NADPH instead of NAD^+ and 90 min reaction time. The reaction was initiated by addition of 40 μg human or 50 μg rat microsome proteins with 0.2 mM NADPH in PBS buffer. A preliminary experiment had determined the velocity of 17β-HSD3 within the linear range in the above conditions. The reactions were stopped by adding 2 ml ice-cold ether. The steroids were extracted and the organic layer was dried under nitrogen. The steroids were separated chromatographically on thin-layer plates in chloroform and methanol (97:3, v/v) and the radioactivity was measured using a scanning radiometer. The percentage conversion of androstenedione to testosterone was calculated by dividing the radioactive counts identified as testosterone by the total counts associated with androstenedione plus testosterone.

2.5. Measurement of 3β-HSD and 17β-HSD3 activities ex vivo

(+)- and (−)-Gossypols (20 mg/(kg day)), dissolved in corn oil, were fed orally to 90-day-old male Sprague–Dawley rats (four per group) for a week. Control animals were fed with the corn oil vehicle. All animals were on standard chow diet. The dose was based on the effectiveness of racemic gossypol as male contraceptive in male rats [3]. Three hours after the last dose, testes were collected and cut into 100 mg pieces and exposed to 5 μM [^{3}H]pregnenolone (for 3β-HSD) or 1 μM [^{3}H]androstenedione (for 17β-HSD). The 1 ml *ex vivo* assay mixtures containing 5% FCS (Sigma–Aldrich) and 0.1 mM PBS was incubated at 37 °C in a shaking water bath (75 rpm) for 15 min. The incubation time was preliminarily determined to be within linearity of these enzyme activities. The extraction, separation and detections of steroids were performed according to above-mentioned methods for both enzymes.

2.6. Statistics

Assays were repeated twice. The IC_{50} was calculated using GraphPad version 4.0 (GraphPad Software Inc., San Diego, CA) using nonlinear regression of curve fit with one-site competition. Lineweaver–Burk plot was used to determine the mode of inhibition. All data are expressed as means ± SEM. The comparison of IC_{50}s between gossypol enantiomers or between species for each enzyme was performed by the Student t test. The *ex vivo* inhibition data were analyzed by one-way ANOVA with multiple comparisons performed by the Duncan multiple range test to identify differences between groups. Differences were considered to be significant if P was less than or equal to 0.05.

3. Results

3.1. Effects of gossypol enantiomers on 3β-HSD activities in human and rat testicular microsomes

3β-HSD catalyzes the step of progesterone biosynthesis from pregnenolone in Leydig cells. As shown in Table 1 and Fig. 1,

Table 1
IC_{50} of (+)-gossypol and (−)-gossypol inhibiting human and rat 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase 3 (17β-HSD3).

Chemicals	IC_{50} (μM)					
	3β-HSD			17β-HSD3		
	Human	Rat	P^a	Human	Rat	P^a
(+)-Gossypol	4.45 ± 0.50	0.19 ± 0.05	0.0002	1.13 ± 0.12	10.93 ± 2.27	0.0051
(−)-Gossypol	2.77 ± 0.19	0.23 ± 0.03	0.0001	0.36 ± 0.09	3.43 ± 0.46	0.0006
P^b	0.021	0.547		0.002	0.018	

Mean ± S.E.M., $n = 4$.
[a] Comparison between species.
[b] Comparison between gossypol enantiomers.

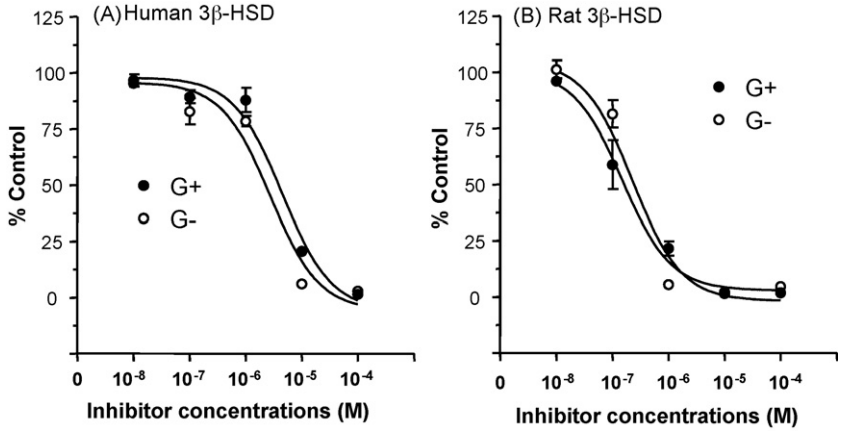

Fig. 1. (A and B) Inhibition of gossypol enantiomers on human and rat testicular 3β-hydroxysteroid dehydrogenase (3β-HSD). 30 μg (for human) or 6 μg (for rat) of testis microsomes were incubated with different concentrations of (+)-gossypol (G+) and (−)-gossypol (G−) and 200 nM pregnenolone in the presence of 0.2 mM NAD^+ for 30 min. Values from four samples in a duplicate assay are represented.

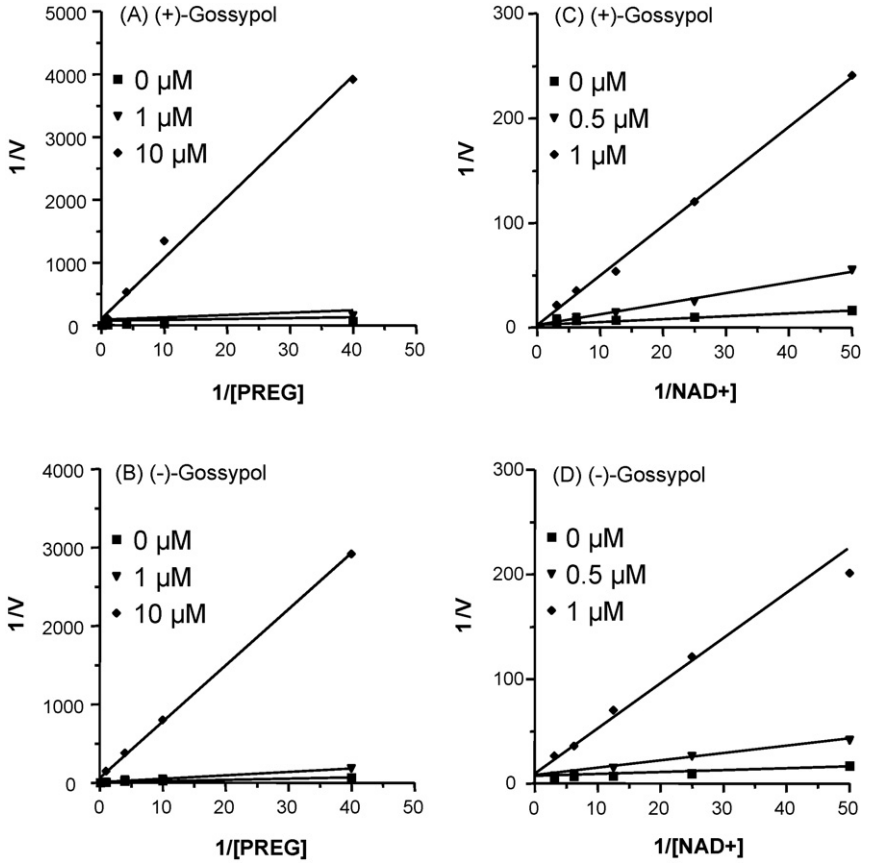

Fig. 2. The mode of inhibition of gossypol enantiomers on rat testicular 3β-hydroxysteroid dehydrogenase (3β-HSD). Lineweaver–Burk plots were drawn for (+)-gossypol (G+, Panel A) and (−)-gossypol (G−, Panel B) in the inhibition of 3β-HSD after using different pregnenolone concentrations, and those for G+ (Panel C) and G− (Panel D) after using different NAD^+ concentrations. Values from four samples in a duplicate assay are represented.

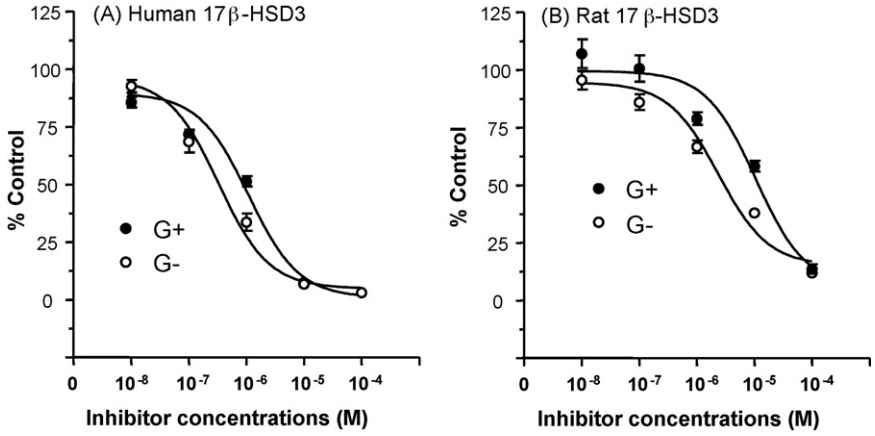

Fig. 3. (A and B) Inhibition of gossypol enantiomers on human and rat testicular 17β-hydroxysteroid dehydrogenase 3 (17β-HSD3). 40 μg (for human) or 50 μg (for rat) of testis microsomes were incubated with different concentrations of (+)-gossypol (G+) and (−)-gossypol (G−) and 200 nM androstenedione in the presence of 0.2 mM NADPH for 90 min. Values from four samples in a duplicate assay are represented.

gossypol enantiomers were 3β-HSD inhibitors. They inhibited human 3β-HSD within micromolar range (Fig. 1 and Table 1). For human 3β-HSD, (−)-gossypol was slightly more potent than the (+) enantiomer ($P < 0.05$). However, there was a difference between species, with more potent inhibition on rat 3β-HSD with IC_{50} around 0.2 μM. The Lineweaver–Burk plot showed that both enantiomers were competitive inhibitors of rat testicular 3β-HSD (Fig. 2A and C). When different cofactor (NAD^+) concentrations were used (Fig. 2B and D), the Lineweaver–Burk plot showed that both gossypol enantiomers competed with the cofactor binding site of rat testicular 3β-HSD. These data indicate that both gossypol enantiomers are competitive inhibitors of 3β-HSD via the NAD^+ binding site of the enzyme.

3.2. Effects of gossypol enantiomers on 17β-HSD3 activities in human and rat testis microsomes

17β-HSD3 catalyzes the last step in testosterone biosynthesis from androstenedione in Leydig cells. As shown in Table 1 and Fig. 3, gossypol enantiomers were 17β-HSD3 inhibitors. For human 17β-HSD3, (−)-gossypol was more potent than the (+) enantiomer with IC_{50} of 0.36 μM ($P < 0.01$). However, there was a difference between the human and rat samples, with less potent inhibition of the rat 3β-HSD with a 9-fold decrease in IC_{50}. Again, (−)-gossypol was more potent than the (+) enantiomer (Table 1). The Lineweaver–Burk plot showed that both enantiomers were competitive inhibitors of rat testicular 17β-HSD3 (Fig. 4A and C). When

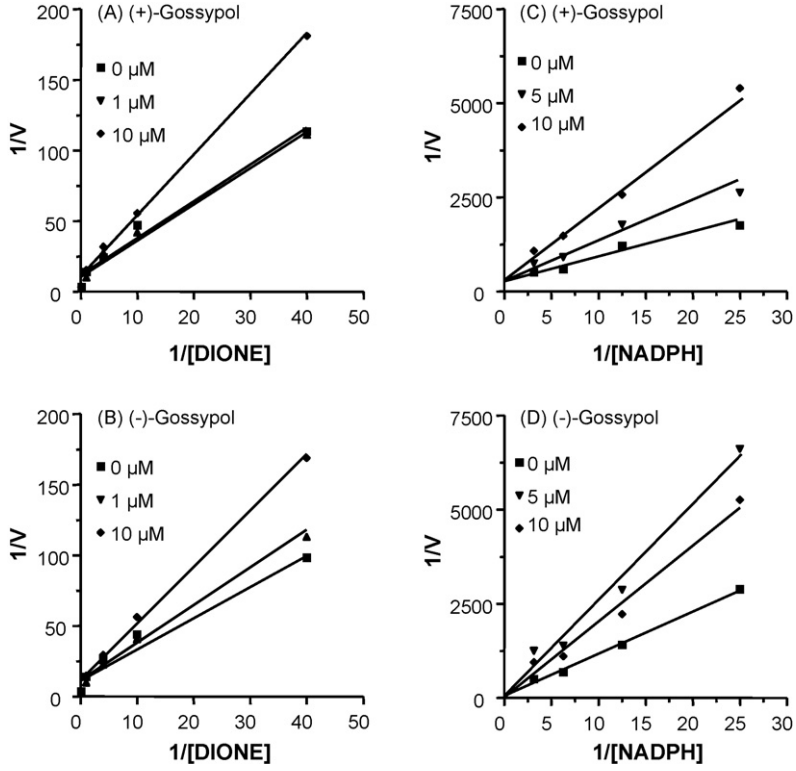

Fig. 4. The mode of inhibition of gossypol enantiomers on rat testicular 17β-hydroxysteroid dehydrogenase 3 (17β-HSD3). Lineweaver–Burk plots were drawn for (+)-gossypol (G+, Panel A) and (−)-gossypol (G−, Panel B) in the inhibition of 17β-HSD3 after using different androstenedione concentrations, and those for G+ (Panel C) and G− (Panel D) after using different NADPH concentrations. Values from four samples in a duplicate assay are represented.

different cofactor (NADPH) concentrations were used (Fig. 4B and D), the Lineweaver–Burk plot showed that both gossypol enantiomers competed with the cofactor binding site of rat testicular 17β-HSD3. These data indicate that both gossypol enantiomers are competitive inhibitors of 17β-HSD3 via the NADPH binding site of the enzyme.

3.3. Effects of gossypol enantiomers on 3β-HSD and 17β-HSD3 activities in vivo

Treatment of rats with (+)- or (−)-gossypol for 1 week resulted in a significant inhibition of 3β-HSD activity in testes ($P < 0.05$). However, no inhibition of 17β-HSD was detected.

4. Discussion

This is the first study to examine the effects of gossypol enantiomers on human and rat 3β-HSD and 17β-HSD3 activities. Because 3β-HSD and 17β-HSD3 are almost exclusively expressed in Leydig cells, inhibition of both enzymes may be used as androgen deletion treatment of metastatic prostate cancer.

In a previous study, racemic gossypol inhibited the conversion of pregnenolone into progesterone in bovine luteal cells [8]. In this study, we demonstrated that both (+)- and (−)-gossypols inhibited 3β-HSD activities. In addition, we observed different levels of inhibition by species: rat 3β-HSD was more sensitive to the inhibitory effects of gossypol enantiomers and both enantiomers had similar potencies (Table 1). This inhibition was also observed in the *in vivo* study (Fig. 5).

We also demonstrated (−)-gossypol as a potent inhibitor of human 17β-HSD3 with IC_{50} of 0.36 μM (Table 1). The inhibition of 17β-HSD3 by gossypol varied by species and by the enantiomer used. For example, human 17β-HSD3 was more sensitive to inhibition by gossypol enantiomers. Ninefold lower concentrations of gossypol enantiomers were needed to inhibit human 17β HSD3 compared to rat 17β HSD3. (−)-Gossypol was three times more potent as an inhibitor of both human and rat 17β-HSD3 than (+)-gossypol. Inhibition of 3β-HSD and 17β-HSD3 did not correlate with the other actions of gossypol: only (−)-gossypol suppressed spermatogenesis and inhibited BCL-2 [4]. We observed a more potent inhibition of human 17β-HSD3 with (−)-gossypol. Although gossypol enantiomers inhibited rat 17β-HSD3 *in vitro*, *ex vivo* study did not show the inhibition by these compounds at the given dose when compared to 3β-HSD. Therefore, the given dose of gossypol enantiomers may not be enough to reach inhibitory concentration for rat 17β-HSD3. However, as the gossypol enantiomers are 7 times more potent for human 17β-HSD3 than for rat 17β-HSD3, *in vivo* inhibition might be achieved because the IC_{50} of (−)-gossypol for human 17β-HSD3 (0.36 μM) was as low as for rat 3β-HSD (0.23 μM).

The Lineweaver–Burk plot analysis in the presence of gossypol enantiomers with various concentrations of substrates pregnenolone (for 3β-HSD, Fig. 2) or androstenedione (for 17β-HSD3, Fig. 4) showed that gossypol enantiomers were competitive inhibitors of both rat 3β-HSD and 17β-HSD3. Further analysis showed that gossypol enantiomers competed with cofactor NAD^+ (for 3β-HSD) or NADPH (for 17β-HSD3). This competitive inhibition with NAD^+ by gossypol has been observed in another dehydrogenase, lactate dehydrogenase isoenzyme 4 in the sperm [4]. Another study showed that gossypol derivatives had a similar inhibitory mechanism on 17β-HSD isoform 1 [14]. Gossypol enantiomers have also been found to be competitive inhibitors of 11β-hydroxysteroid dehydrogenase (see reviews [15]).

The (−)-gossypol enantiomer potently inhibited human 17β-HSD3 with IC_{50} of 0.36 μM. The IC_{50}s of gossypol enantiomers inhibiting human 17β-HSD3 are within the blood levels of gossypol in clinical use as a male contraceptive. A recent study using low doses (7.5 mg/day) of racemic (±)-gossypol showed that blood gossypol levels reached 186 ± 113 ng/ml (about 0.36 ± 0.22 μM) after 12 weeks of treatment [16]. The large-scale clinical trials performed in 1980 used much higher gossypol doses (20 mg/day) which may result in much higher blood gossypol levels [3]. However, most subjects in clinical trials had T levels in the normal range after gossypol treatment, except in patients with significantly reduced serum T levels due to gossypol-induced hypokalemia [3]. Therefore, a higher dose than that used for male contraceptives may be required to reduce T levels as treatment for prostate cancer. However, recent studies have shown over 30-fold increase in 17β-HSD3 mRNA levels [17] and up-regulation of 17β-HSD3 after 48-h application of dutasteride, a 5α-reductase inhibitor, in LNCaP, an androgen-receptor positive cell line [18]. A polymorphism in the human 17β-HSD3 gene (*HSD17B3*, G289S) has been associated with an increased susceptibility to prostate cancer [19]. In this regard, gossypol enantiomers, especially the (−) enantiomer, may be used to inhibit 17β-HSD3 in prostate cancer cells, thus eliminating the production of T *de novo* in prostate cancer cells.

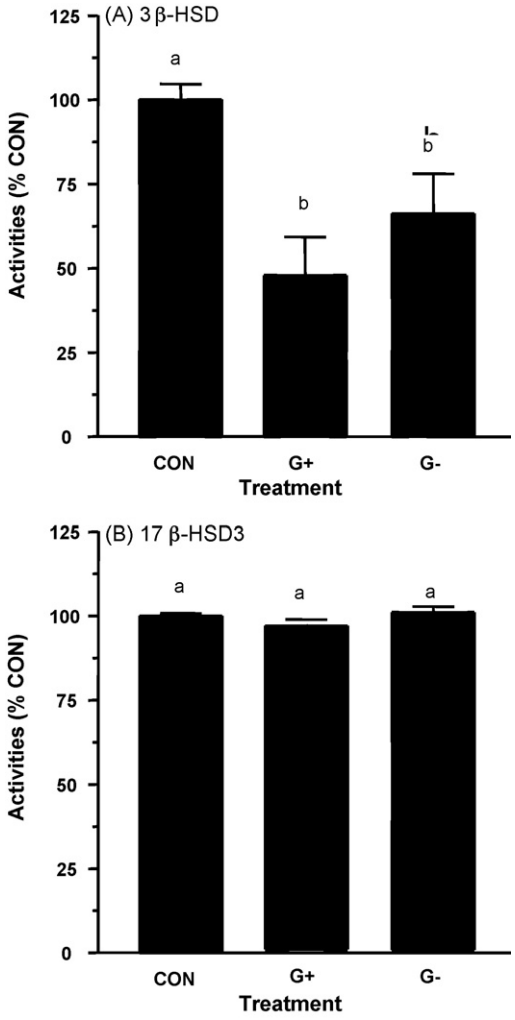

Fig. 5. Effects of gossypol enantiomers on rat testicular 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase 3 (17β-HSD3) *in vivo*. Adult male rats were fed orally with vehicle control (CON) or 20 mg/kg daily of (+)-gossypol (G+) as well as (−)-gossypol (G−) for 1 week. 3β-HSD (Panel A) and 17β-HSD3 (Panel B) were measured *ex vivo* as described in Section 2. Mean ± S.E., $n = 4$. Different letters indicates that there was significant difference between two groups at $P < 0.05$.

The (−)-gossypol enantiomer strongly inhibits Bcl-2 and might be used in the future to treat prostate cancer [20]. The potent inhibition of 17β-HSD3 may be another benefit of (−)-gossypol in the treatment of prostate cancer. In addition, the effects of (−)-gossypol were enhanced by castration [11]. Sensitivity to gossypol is species- and enantiomer-specific. For example, human 17β-HSD3 was 9-fold sensitive to the inhibition of (−)-gossypol. These differences should be considered in future studies of gossypol.

In conclusion, the present study demonstrated (+) and (−)-gossypols as potent inhibitors of testicular 17β-HSD3 *in vitro* with differences by species and by enantiomers.

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