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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,