Gossypol inhibits estrogen binding to rat / -fetoprotein

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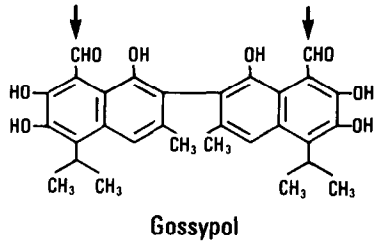
We find that gossypol, a male anti-fertility compound, is a reversible competitive inhibitor of estrogen binding to rat α-fetoprotein (AFP). The K_d of gossypol for rat AFP is 1.75 μM, which is similar to gossypol's affinity for lactate dehydrogenase isozyme X, the putative site where gossypol exerts its anti-fertility effects. Reacting sodium cyanoborohydride with gossypol reduces its affinity for AFP, showing that intact aldehyde groups on gossypol are important for binding to rat AFP and indicating that gossypol is specifically interacting with a nucleophilic site on AFP that influences estrogen binding.

α-Fetoprotein *Estrogen* *Gossypol*

1. INTRODUCTION

Gossypol (1,1′,6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dicarboxyaldehyde) (fig. 1), a polyphenolic compound, is the principal pigment in cottonseed [1]. There is considerable interest in gossypol because it has been successfully used as a male contraceptive in large clinical trials in China [2–4]. The mechanism of action for this male anti-fertility effect is still under investigation [3–7].

I decided to study the effect of gossypol on estrogen binding to rat α-fetoprotein (AFP) because (1) Philipp and Maripuri (unpublished observations) found that gossypol inhibits serine proteases and (2) previous studies have shown that serine protease inhibitors and substrates inhibit estrogen binding to rat AFP [8–10]. Here, I report findings which show that gossypol inhibits estrogen binding to rat AFP, and that gossypol's binding to AFP appears to involve interaction with a nucleophilic site on AFP.



Gossypol

Fig. 1. Structure of gossypol. The arrows indicate aldehyde groups.

2. MATERIALS AND METHODS

The source of rat α-fetoprotein, serum from Buffalo rats with Morris Hepatoma 7777, was a gift from Dr Stewart Sell. Gossypol-acetic acid was purchased from Sigma. Stock solutions of 10 mM gossypol in ethanol at pH 4 were stored at −20°C. Under these conditions gossypol's binding activity for AFP was stable for at least two months.

For the estrogen binding assay, the serum was diluted 1:100000 into buffer containing 20 mM Hepes, 2 mM EDTA, 50 mM NaCl, 20% glycerol (HEG buffer) and 0.2 mg/ml ovalbumin. This dilution to a final AFP concentration of about 0.5 nM insures that the AFP concentration is lower than both the [^{3}H]estrone ([^{3}H]E_1] concentration in the binding assay and the equilibrium dissociation constant (K_d) of E_1 for AFP. Both conditions are necessary to obtain interpretable data from our experiments [11]. The competitive binding assay was begun by incubating the AFP solution in glass tubes on ice with 1 nM [^{3}H]estrone (85–115 Ci/mmol, New England Nuclear), alone, or with gossypol, or with a 300-fold excess of unlabelled

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estrone for 2 1/2 h. Bound steroid was separated from unbound steroid using a dextran-coated charcoal technique [9] which involves incubation of 2 ml sample solution with 0.2 ml of 100 mg/ml charcoal, 10 mg/ml dextran, and 5 mg/ml ovalbumin for 1 min and then centrifugation at $6000 \times g$ for 15 min to remove the charcoal. The radioactivity in 1 ml of supernatant was determined in a liquid scintillation counter. Specifically bound estrone was determined by subtracting the amount of [^{3}H] estrone bound in the presence of a 300-fold excess concentration of non-radioactive estrone from the amount of [^{3}H]estrone bound in the absence of the non-radioactive estrone.

3. RESULTS AND DISCUSSION

Fig. 2 shows that μM concentrations of gossypol compete with 10^{-9} [^{3}H]estrone (E_1) for binding to rat AFP. A more quantitative determination of gossypol's affinity for rat AFP was obtained using competitive Scatchard analysis [12,13]. The Scatchard graph presented in fig. 3 shows that in the presence of gossypol the affinity of [^{3}H]E_1 binding to rat AFP decreases while the maximal number of binding sites remains unchanged. This indicates

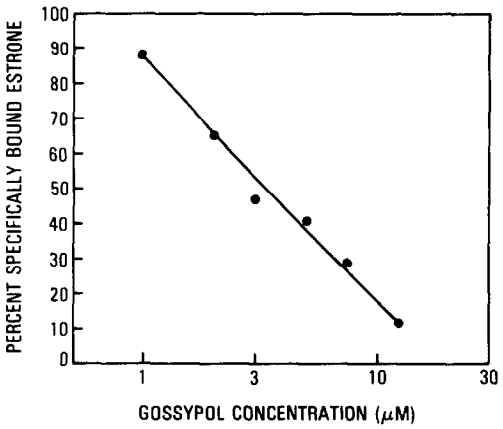

Fig. 2. Competition of gossypol with [^{3}H]estrone for rat α-fetoprotein. Rat AFP was incubated with 1×10^{-9} M [^{3}H]E_1 alone or with different concentrations of gossypol, or a 3×10^{-7} M unlabeled estrone at 0°C, pH 7.8 for 2 1/2 h. Then [^{3}H]estrone specifically bound to AFP was determined using the dextran-coated charcoal technique. Specifically bound estrone in the control sample was 18 000 cpm/ml.

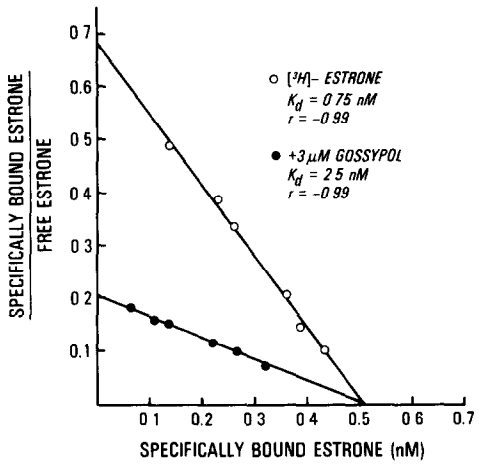

Fig. 3. Scatchard analysis of the effect of 3×10^{-6} M gossypol on the binding of [^{3}H]estrone to rat AFP. AFP was incubated with [^{3}H]E_1 (5×10^{-10} M to 4.5×10^{-9} M) ± unlabeled estrone or 3×10^{-6} M gossypol for 2 1/2 h at 0°C, pH 7.8. Bound steroid was separated from unbound steroid using dextran-coated charcoal. Ordinate: specifically bound [^{3}H]estrone (cpm/ml). Abscissa: specifically bound [^{3}H]estrone (nM).

that gossypol is a reversible competitive inhibitor of estrogen binding to AFP.

One can calculate K_d^{gossypol}, the equilibrium dissociation constant of gossypol for rat AFP, from the equation:

$$K_d^{\text{gossypol}} = \frac{K_d C}{K_d^{\text{app}} - K_d}$$

where K_d is the equilibrium dissociation constant of [^{3}H]E_1 for rat AFP, K_d^{app} is the apparent equilibrium dissociation constant of [^{3}H]E_1 in the presence of gossypol, and C is the gossypol concentration [13]. This equation gives an upper limit on K_d^{gossypol} because the gossypol concentration may be less than the nominal concentration due to factors such as association of gossypol with other proteins and decomposition of gossypol during the binding assay. Using that equation we find that K_d^{gossypol} for rat AFP is 1.75 μM (table 1).

Authors in [7] suggested that gossypol acts by selectively inhibiting the lactate dehydrogenase (E.C.1.1.1.27) isozyme X (LDH X), which plays an important role in sperm metabolism. Consistent with the need for high concentrations of gossypol (20 mg/day) to achieve contraception [2–4], the K_i

Table 1
Effect of gossypol on estrone binding to rat α-fetoprotein

Condition	K_d (M)	$K_d^{gossypol}$ (M)
[^{3}H]estrone ($n=7$)	8×10^{-10}	
$+3\times10^{-6}$ M gossypol ($n=7$)	21.7×10^{-10}	1.75×10^{-6}

n = number of experients

of gossypol for LDH X is between 2–5 μM [7,14]. This affinity is comparable to that of gossypol for rat AFP (table 1) and for human testosterone–estrogen binding globulin (unpublished). This raises the possibility that in bioassays gossypol may be inhibiting the binding of steroid hormones to proteins.

Are aldehyde groups on gossypol important in its binding to AFP? A reason for thinking that the aldehyde groups on gossypol would be important for its binding to AFP comes from a hypothesis based on the observation that AFP contains a site that both recognizes serine protease inhibitors and substrates and influences estrogen binding [8–10]. This hypothesis, that rat AFP contains a nucleophilic (electron-rich) site that influences estrogen binding by interacting with electrophilic sites on steroids and/or with proton donors on rat AFP, predicts that the electrophilic (electron-deficient) aldehyde groups on gossypol (fig. 1) would be important for its binding to rat AFP [15]. To test this hypothesis we incubated gossypol with the reducing agent sodium cyanoborohydride ($NaBH_3CN$) a chemical that selectively converts aldehyde groups to alcohols [16]. Table 2 shows that incubation of gossypol with $NaBH_3CN$ significantly reduced gossypol's affinity for rat AFP supporting this hypothesis. This finding suggests that: (1) inhibition of [^{3}H]E_1 binding to AFP by gossypol involves a specific interaction between gossypol and rat AFP rather than being due to nonspecific hydrophobic partitioning of gossypol into AFP, and (2) changing the electrophilicity of sites on gossypol may prove useful in regulating its binding to AFP.

Table 2
Incubation of gossypol with sodium cyanoborohydride decreases gossypol's affinity for rat α-fetoprotein

Condition	Specifically bound [^{3}H]E_1 (cpm/ml)
Control	16560
+ 5 μM gossypol	5620
+ 5 μM gossypol treated with $NaBG_3CN$	12240

A solution of 10 mM gossypol acetic acid in ethanol (pH 4) and 10 mM sodium cyanoborohydride was incubated for 30 min at 22°C. Then the $NaBH_3CN$ concentration was increased to 20 mM and the incubation continued for 6 h at 22°C. Treated and untreated gossypol were tested for their ability to inhibit [^{3}H]E_1 binding to rat AFP as described in section 2.

Concerning the latter possibility, two modifications that could increase the electrophilicity of the aldehyde groups would be substituting either a nitro group for the isopropyl group or a ring nitrogen para to the aldehyde group. There is precedence for these modifications to increase the aldehyde group's electrophilicity in the chemistry of pyridoxal phosphate [17].

Gossypol appears to have minimal side effects. This suggests an interesting and unexpected application of this work, the use of gossypol and possibly the gossypol analogues described above to study the function of AFP in rats and mice, including male rats with AFP-secreting hepatomas.

Also, it will be important to find out if 'NaBH$_3$CN-treated' gossypol possesses contraceptive activity, anti-herpes activity [18], or if it inhibits lactate dehydrogenase isozyme X [7], serine enzymes, or the growth of *Trypanosoma cruzi* [14]. If reduced gossypol is ineffective in those assays, then the previously described gossypol analogues may have application in those systems.

While this research was being completed, it was reported [19] that gossypol bound to human serum albumin with high affinity ($K_d \sim 10^{-7}$ M) at the bilirubin binding site. The effect of $NaBH_3CN$ treatment of gossypol on its binding to albumin was not studied. The presence of a site that binds gossypol on both albumin and AFP might be expected, since AFP is thought to have evolved by a duplication of the albumin gene [20–22], and both proteins have some ligand binding properties in common, including binding bilirubin [23–26]. However, the report [26] that bilirubin does not inhibit estrogen binding to AFP suggests that there are differences between AFP and albumin in their binding of gossypol.

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