Inhibition of testicular LDH-X from laboratory animals and man by gossypol and its isomers

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Summary. The inhibitory effect of (+)-, (–)-, (±)-gossypol and (±)-gossypol acetic acid upon testicular cytosolic LDH-X was measured *in vitro*. Gossypol acetic acid (0–100 µmol/l) inhibited LDH-X prepared from the testes of the mouse > rabbit > human > rat > hamster. There was no relationship between inhibition and in-vivo antifertility activity. LDH activity measured *in vitro* in serum of men and hamsters was unaffected by gossypol. Gossypol and its isomers were non-competitive inhibitors of human and hamster LDH-X with respect to the coenzyme NADH, competitive inhibitors of human LDH-X and noncompetitive–competitive inhibitors of hamster LDH-X with respect to the substrate α -ketobutyrate. Co-incubation with human serum albumin or poly-L-lysine but not lysine protected human and hamster LDH-X from gossypol.

Introduction

Gossypol, a polyhydroxylated binaphthalene extracted from the cotton plant, is an effective oral male contraceptive. Infertility, associated with immotile spermatozoa, subsequent azoospermia and few side effects, has been reported from large clinical trials in China and smaller studies in Austria and Brazil (see Segal, 1985). In spite of this clinical application the antifertility mechanism remains unknown. Recent experiments suggest that the antifertility properties of gossypol are associated specifically with the (–)-isomer (Waller *et al.*, 1983; Matlin *et al.* 1985). The apparent selectivity of gossypol towards mature and developing spermatozoa suggests that they possess a unique target. Whilst many of the cellular and biochemical properties of the testis may be found elsewhere in the body, lactate dehydrogenase (LDH: EC 1.1.1.27) is of interest. A specific isoenzyme LDH-X is found only in germ cells, appearing first in pachytene spermatocytes (Sarkar, Dubey, Banerji & Shah, 1978). LDH-X is essential for the metabolic activities in cells in the later stages of spermatogenesis (Jutte, Grootegoed, Rommerts & van der Molen, 1981). That inhibition of this enzyme may account for the antifertility activity of gossypol was first suggested by Lee & Mallings (1981) and numerous reports have confirmed this in-vitro observation (Lee, Moon, Yuan & Chen, 1982; Tso & Lee, 1982; Giridharan, Bamji & Sankaram, 1982; Eliasson & Virji, 1983; Olgiati & Toscano, 1983; Hoffer, 1985). However, many testicular and non-testicular enzymes can be inhibited by gossypol which raises the possibility that this interaction may not be related to the antifertility properties of gossypol (see Segal, 1985). This paper describes the interaction of gossypol with LDH-X prepared from the testes of a variety of species, susceptible and unsusceptible, to the antifertility effects of gossypol and attempts to try and elucidate the importance of the in-vitro inhibition.

Materials and Methods

Racemic gossypol acetic acid (a gift from N.I.H., U.S.A.) was routinely recrystallized (melting point 183°C) from ethyl acetate and stored dry in the dark until immediately before use. Gossypol, as the ($\pm$)-, (+)- and (–)-isomers, was prepared as described elsewhere (Matlin *et al.*, 1985). The doses and concentrations always refer to the content of the binaphthyl. Gossypol was diluted in ethanol and added directly to the assay tubes, while controls received ethanol alone (final concentration 1.7% v/v).

Testes were stored whole in liquid nitrogen until used. Human testes were obtained from patients (aged 58–65 years) undergoing surgery for inguinal hernia; spermatogenesis was normal when examined histologically in frozen sections. Testes were obtained from rabbits, rats (Sprague–Dawley) and mice (Swiss albino), which were not susceptible to the antifertility effect of gossypol, and the golden hamster which is susceptible to gossypol (see below for further details). Cytosol fractions were prepared according to the method described by Giridharan *et al.* (1982) and stored in liquid nitrogen until required for assay. Serum from men and adult hamsters was also prepared as a source of the other LDH-isoenzymes.

LDH-X assay conditions were based upon selective substrate utilization as described by Schatz & Segal (1969). Substrates (Sigma Chemical Co. Ltd, Poole, Dorset, U.K.), pyruvate, α -ketobutyrate, α -ketovalerate, α -ketoglutarate and NADH, were prepared in sodium potassium phosphate buffer (100 mmol/l, pH 7.4). Optimization of enzyme, and substrate concentrations as well as specificity towards LDH-X was as indicated by Wilkinson & Withycombe (1965), Lee *et al.* (1982), Tso & Lee (1982), Giridharan *et al.* (1982), Eliasson & Virji (1983), Olgiati & Toscano (1983) and Hoffer (1985) and demonstrated in preliminary experiments. Enzyme activity was observed at 37°C by the change in absorbance of NADH at 340 nm using a Shimadzu recording spectrophotometer. Cytosols were diluted to achieve a linear plot to estimate rate of change.

Results

The in-vivo antifertility effects of gossypol in man and the lack of antifertility action in the rabbit are reported elsewhere (see Segal, 1985). As fertility studies have not previously been reported from our laboratories a brief description follows (for further details see Higgins (1985) and Higgins & Morris (1985)). Rats received oral gossypol (20 mg/kg daily); 4 out of 6 rats died between 10 and 24 days of treatment associated with profound loss of body weight. Histological examination of the testes showed no abnormalities. Haematocrit and haemoglobin concentrations decreased by 50%. Mice received orally between 6 and 100 mg gossypol/kg daily. The mice treated with 6, 12 and 25 mg/kg survived treatment, mated normally and fertility in mated female mice was measured every 7 days for 63 days. Litter sizes were not changed. Hamsters treated with 5 mg gossypol/kg (by mouth, daily) for 35 days but not for 20 days and with 20 mg/kg for 20 days were significantly subfertile; hamsters treated with 20 mg/kg for 35 days were completely infertile. Infertility was assessed by implantation sites in mated females. Sperm motility *in vitro* was reduced. Testicular weight was unchanged at 20 mg/kg but raised in the 5 mg/kg group; other organ weights were unchanged. Histology revealed a disorganized spermatogenic epithelium only after 35 days of treatment with 20 mg/kg.

Concentration-dependent inhibition of LDH-X activity was demonstrated for all the enzyme activities examined (Fig. 1). The susceptibility of the testicular cytosolic enzyme to gossypol was mouse > rabbit > human > rat > hamster. The EC-50 values ranged from 15 μ mol/l (mouse) to 70 μ mol/l (hamster). LDH activity in the serum of man and hamster was measured using pyruvate (1 mmol/l) and NADH (17 mmol/l) as the substrates in the presence of 10, 50 or 100 μ mol gossypol/l. No inhibition of enzyme activity was seen at these concentrations.

Fig. 1. Effect of gossypol upon testicular LDH-X *in vitro*. Gossypol was preincubated with testicular cytosol for 15 min before the addition of substrates. The substrate concentrations were 1 mmol α -ketobutyrate/l and 0.17 mmol NADH/l for hamster, man and rabbit and 4.1 mmol α -ketoglutarate/l and 0.17 mmol NADH/l for rat and mouse. The results are the mean of at least 3 separate determinations.

Fig. 2. Kinetics of the inhibition of testicular LDH-X activity by gossypol (50 μ mol/l). Cytosols were preincubated for 15 min with gossypol before addition of the substrate. Incubates in which α -ketobutyrate concentrations were varied contained 0.17 mmol/l NADH. Incubates in which NADH concentrations were varied contained 1 mmol/l α -ketobutyrate. Each point is the mean of 3 observations. ■, Control; ▲, (-)-gossypol; △, (+)-gossypol; ○, (±)-gossypol; ●, (±)-gossypol acetic acid.

The kinetics of LDH-X inhibition by gossypol preparations (50 $\mu\text{mol/l}$) were examined (Fig. 2). In human and hamster testicular cytosols the inhibition by gossypol was noncompetitive with respect to NADH. Gossypol decreased the V_{max} by $\sim 50\%$. Little if any change was noted for K_m . The inhibition of hamster LDH-X was more complex when using α -ketobutyrate as the substrate and can be described as mixed inhibition, competitive–noncompetitive. The results indicate that (–)-gossypol is a more potent inhibitor of hamster LDH-X than is the racemate or the (+)-isomer when using α -ketobutyrate as the substrate. The inhibition of human LDH-X by gossypol using α -ketobutyrate as the substrate was competitive.

When the inhibitory effects (% of control, mean $\pm$ s.e.m., $n = 3$) of (±)-gossypol acetic acid (50 $\mu\text{mol/l}$) upon human (60 ± 8) and hamster (31 ± 4) testicular LDH-X were tested by addition of human serum albumin (HSA), poly-L-lysine (50 $\mu\text{mol/l}$) and L-lysine (100 $\mu\text{mol/l}$) 15 min before inclusion of 1 mmol α -ketobutyrate/l and 0.17 mmol NADH/l, the values for human and hamster respectively were: 100 ± 1 and 100 ± 1 with 0.4 mg HSA/ml, 70 ± 1 and 43 ± 2 for gossypol + 0.1 mg HSA/ml, 96 ± 1 and 80 ± 1 for gossypol + 0.4 mg HSA/ml, 98 ± 2 and 102 ± 1 for poly-L-lysine, 84 ± 1 and 91 ± 1 for gossypol + poly-L-lysine, 98 ± 1 and 99 ± 1 for lysine, and 63 ± 1 and 36 ± 1 for gossypol + lysine.

Discussion

Gossypol (0–100 $\mu\text{mol/l}$) produced a concentration-dependent inhibition of testicular cytosol LDH-X for all species examined. The EC-50 concentrations which ranged from 15 to 70 $\mu\text{mol/l}$ are very similar to those reported by others (Lee *et al.*, 1982; Tso & Lee, 1982; Giridharan *et al.*, 1982; Eliasson & Virji, 1983; Olgiati & Toscano, 1983; Hoffer, 1985), but there is a great deal of variation of the reported values which confuses the significance of such estimations. The present study was undertaken to avoid comparisons between laboratories and to see whether there was a relationship between antifertility effects *in vivo* and *in-vitro* inhibition of LDH-X. No relationship could be observed; for instance the mouse was most and the hamster one of the least susceptible to LDH-X inhibition by gossypol in complete contrast to our antifertility data. Some degree of selectivity for the testicular LDH-X appears to have been demonstrated in the present experiments as gossypol failed to inhibit LDH in the serum. These results would support the observation of the selective inactivation of testicular LDH-X reported by Lee *et al.* (1982), although this has been questioned by Hoffer (1985).

The kinetics of the inhibitory effect of gossypol upon LDH-X were examined in two susceptible species, man and hamster, but the type of inhibition was different and would not be consistent with a common *in-vivo* mechanism of action. Inhibition of hamster and human LDH-X was non-competitive with respect to NADH. This suggests that gossypol may bind to LDH-X and the enzyme substrate complex is unaffected. However, the possibility remains that the inhibition was essentially irreversible which would also appear to reduce the V_{max} . Gossypol inhibited LDH-X prepared from hamster testis by a mixed competitive–noncompetitive inhibition with respect to α -ketobutyrate, which indicates that gossypol binds to the enzyme and enzyme substrate complex as well as competing for the active site. Inhibition of human testicular LDH-X was competitive with respect to α -ketobutyrate, suggesting that gossypol competes with the substrate for the active site. Similar complex patterns of *in-vitro* inhibition have been reported for rat and cattle testicular LDH-X (Lee *et al.*, 1982; Giridharan *et al.*, 1982). In all but one experiment there is no suggestion of increased potency of any gossypol sample or isomer on the inhibition of LDH-X *in vitro*. However (–)-gossypol was slightly more potent upon hamster LDH-X when α -ketobutyrate was the substrate and it is this isomer that is believed to possess more potent antifertility activity (Matlin *et al.*, 1985). In general these results provide evidence to suggest that LDH-X inhibition *in vivo* is not a primary contribution to the antifertility effect produced by gossypol, especially since

LDH-X inhibition *in vitro* is produced by the (+)-isomer which lacks antifertility properties (Waller *et al.*, 1983; Matlin *et al.*, 1985).

One of the numerous chemical reactions of gossypol is the formation of Schiff bases with lysine residues of protein (Conkerton & Frampton, 1959). Inactivation of pig LDH-M4 and mouse LDH-X by pyridoxal-5-phosphate has been suggested to be due to modification of a single lysine residue (Gould & Engel, 1980). Gossypol inhibits pepsinogen by binding to lysine residues so this effect may account for most of the *in-vitro* activity (Wong, Nakagawa & Perlman, 1972). The present experiment would substantiate this. Human serum albumin completely protected the enzyme as did poly-L-lysine but not L-lysine. These observations would also indicate why apparent selectivity was seen in the present experiments. Human serum albumin binds with very high affinity to gossypol, K_D about 1×10^{-9} mol/l (Royer & van der Jagt, 1983), which is several orders of magnitude higher than the affinity of gossypol for LDH-X that can be surmised from the EC-50 concentrations, $15\text{--}70 \times 10^{-6}$ mol/l. Serum contains ~ 34 mg albumin/ml (Diem & Lentner, 1970) and so is present in a large excess and would bind gossypol, possibly by the lysine residues, and so decrease the concentration to which enzymes were exposed. Protein binding may also be relevant to all experiments which use unpurified LDH or indeed any other target, the presence of proteins markedly influencing the potency of gossypol and so contributing to the variability of results in the literature. The role of protein binding by gossypol must be significant and should not be overlooked when designing *in-vitro* or *in-vivo* experiments; it probably contributes to the difficulty in interpreting results such as those presented in this study.

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