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RIBONUCLEOTIDE REDUCTASE: AN INTRACELLULAR TARGET FOR THE MALE ANTIFERTILITY AGENT, GOSSYPOL

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SUMMARY: Gossypol is a yellow phenolic compound which reversibly inhibits spermatogenesis making it one of the few effective male antifertility drugs. The cytotoxic effects of gossypol have been associated with its ability to irreversibly inhibit DNA synthesis by a previously unknown mechanism. The results of this study indicate that gossypol is a potent inhibitor of ribonucleotide reductase the rate limiting enzyme activity in DNA synthesis. Furthermore, in agreement with these enzyme studies, DNA synthesis in a hydroxyurea resistant cell line with high levels of ribonucleotide reductase activity showed increased resistance to gossypol when compared to wild type cells with normal levels of reductase activity. Ribonucleotide reductase is the first specific site of action documented for gossypol which can explain its recently described antiproliferative, cell cycle and toxic effects.

INTRODUCTION: Gossypol is a complex, highly reactive yellow polyphenolic compound isolated from cottonseed oil, and is in relatively common use in several countries as a male contraceptive. The antifertility characteristic of this drug is a result of its ability to inhibit spermatogenesis (1) and in most cases its effects are reversible. Although little is known about its mechanism of action and toxicity gossypol does not appear to be a mutagen (2,3,4) and may not appreciably alter DNA structure (5,6,7). Gossypoly has been reported to cause a drug concentration dependent inhibition of *in vivo* proliferation of Ehrlich ascites tumor cells (4). Furthermore, at low concentrations (10 μg/ml) gossypol specifically inhibits DNA synthesis while having little or no effect on RNA and protein synthesis (7). Recent reports have suggested that gossypol may be useful as an antitumor agent since it irreversibly blocks cells in S phase (7), however, a major drawback is that the range between effective and toxic doses appears to be narrow (4). The polyphenolic structure of gossypol, coupled with its ability to inhibit DNA synthesis (7) and antiproliferative

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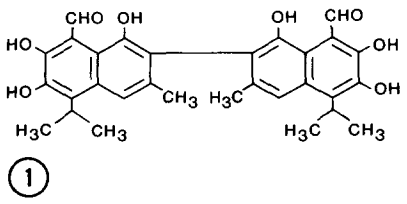
effects (4) suggested to us that this drug may be an inhibitor of ribonucleotide reductase the rate limiting enzyme activity in DNA synthesis.

Ribonucleotide reductase in mammalian cells is composed of two non-identical subunits M1 and M2 (8,9,10). The activity of ribonucleotide reductase is cell cycle dependent, increasing markedly during DNA synthesis (8,9,10) as a result of an increase in the concentration of active protein M2 (11). Protein M2 contains a unique tyrosyl free radical essential for activity (12) which is destroyed by hydroxyurea, and as a result addition of hydroxyurea to growing cells leads to inactivation of protein M2, depletion of deoxyribonucleotide pools and blocked DNA synthesis (13). Over the years our laboratory has isolated and characterized many mutant cell lines showing resistance to the cytotoxic effects of hydroxyurea (10). The objective of this study was to (a) investigate the possibility that gossypol inhibits ribonucleotide reductase activity and (b) determine whether mutant cells selected for resistance to hydroxyurea showed cross resistance to gossypol. Our results indicate that gossypol is indeed a potent inhibitor of ribonucleotide reductase activity thereby establishing, for the first time, a site of action for this drug which can account for its previously documented biological characteristics (4,7).

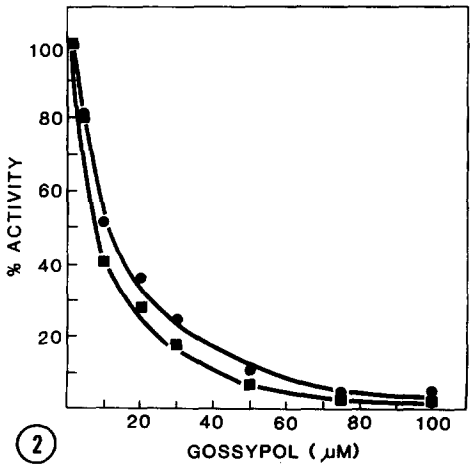
MATERIALS AND METHODS: The mutant cell line used in this study will be described in detail at a later date (manuscript submitted for publication). Briefly, starting with a non-mutagenized population of mouse L cells, a cell line highly resistant to the cytotoxic effects of hydroxyurea was sequentially selected in a stepwise manner in the presence of increasing concentrations of hydroxyurea. The selected mutant was 30-fold more resistant to hydroxyurea (a D_{10} for wild type of 0.17 mM vs a D_{10} for the mutant line of 5.5 mM hydroxyurea) as a result of increased intracellular levels of ribonucleotide reductase activity (wild type reduces 1.0 nmole CDP/3 x 10^6 cells/hr vs 18.5 nmoles CDP/3 x 10^6 cells/hr for the drug resistant line). Cell free extracts used as a source of enzyme for ribonucleotide reductase assays were prepared in the following manner. Wild type and drug resistant mouse L cells were grown on 150 mm plastic tissue culture dishes as previously described (14). Following removal with trypsin solution the cells were washed 3 times with ice cold phosphate buffered saline (PBS), pH 7.5. The final cell pellet was resuspended in a minimal volume of assay buffer (20 mM Tris-HCl pH 7.2, 1 mM DTT) to yield a final density of 2.5 $\pm$ 0.5 x 10^7 cells/ml. The cell suspension was placed in an ice bath and was disrupted by three 10 second periods of sonication. Following sonication the suspension was cleared of cellular debris by centrifugation at 15,000 x g for 15 min. at 4°C. The resulting cell extracts containing 1-3 mg/ml protein were rapidly frozen and kept at -70° C. CDP and ADP ribonucleotide reductase activities in cell free extracts were determined by the methods of Steeper and Stuart (15) and Cory et al (16) respectively, using [^{14}C] CDP and [^{14}C] ADP as substrates and snake venom to hydrolyze the nucleotides

(14). Reactions were carried out for 20 min at 37°C for ADP and CDP reductase and 1 hr at 37°C in the presence of snake venom. The effect of gossypol on DNA synthesis in wild type and hydroxyurea resistant mouse L cells was carried out as follows. Exponentially growing cells were plated at a density of 1.5-2.0 x 10^6 cells per 100 mm tissue culture plate. After incubation at 37°C for 20 hrs the medium was aspirated, the plates washed with sterile PBS then fresh medium supplemented with various concentrations of gossypol was added and the plates were returned to the incubator at 37°C. After 45 minutes [^{3}H] thymidine and cold thymidine were added to final concentrations of 1 μCi/ml and 0.25 μg/ml medium respectively. Incubation was continued at 37°C for 90 minutes then cells were processed for scintillation counting to measure the incorporation of label into DNA (17). Gossypol was obtained from the Sigma Chemical Co. as gossypol acetic acid and since the drug is unstable in aqueous solutions at neutral pH, it was freshly prepared by dissolving in dimethyl sulfoxide or 1 M Na Carbonate, followed by dilution with assay buffer or medium to yield the desired concentrations.

RESULTS AND DISCUSSION: As previously mentioned the polyphenolic structure of gossypol (Fig. 1) in combination with its other reported characteristics (4,7) made it an ideal candidate as an inhibitor of ribonucleotide reductase. To test this hypothesis ribonucleotide reductase activity was determined in the presence of various concentrations of gossypol. The results of these experiments indicated that gossypol is a potent inhibitor of ribonucleotide reductase activity (Fig. 2) and as a result a more detailed kinetic study of the effect of gossypol on the reduction of CDP and ADP was carried out. As shown in Figures 3 and 4 gossypol has little or no effect on the apparent Vmax values but



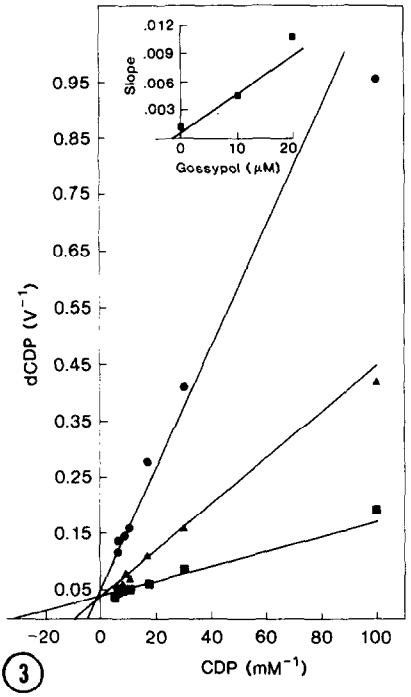
(1)



(2)

Figure 1: The chemical structure of gossypol.

Figure 2: Ribonucleotide reductase activity measured in mouse L cell extracts in the presence of various concentrations of gossypol; CDP reduction (■) and ADP reduction (●).



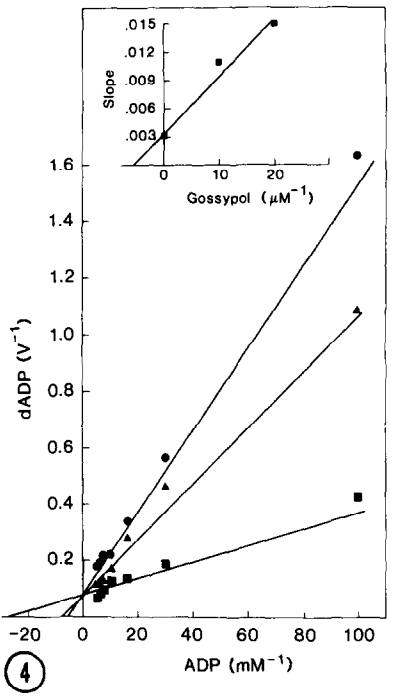


Figure 3: Double reciprocal plots of velocity against CDP concentrations in mouse L cell extracts at several fixed levels of gossypol. Activity in the absence (■) or presence of 10 μM (▲) and 20 μM (●) gossypol. Inset: replot of slopes against drug concentrations.

Figure 4: Double reciprocal plots of velocity against ADP concentrations in mouse L cell extracts at several fixed levels of gossypol. Activity in the absence (■) or presence of 10 μM (▲) and 20 μM (●) gossypol. Inset: replot of slopes against drug concentrations.

dramatically altered the Km values of the two substrates, suggesting that gossypol inhibits the reduction of purines and pyrimidines in a competitive manner. Replots of the slopes versus gossypol concentrations indicated that the Ki values for CDP and ADP reduction were 1.72 μM and 5.5 μM gossypol respectively (inserts Fig. 3 and 4). These values are 1-2 orders of magnitude lower than those reported for other important ribonucleotide reductase inhibitors such as hydroxyurea (14,19), guanazole (19) N-carbamoyloxyurea (17) and pyrazoloimidazole (20), indicating that gossypol is one of the more potent inhibitors of ribonucleotide reductase. In fact gossypol is almost as potent an inhibitor as the very toxic thiosemicarbazones (20,21).

In the past we have found that cells selected for resistance to hydroxyurea are often cross resistant to other drugs (eg. guanazole) that inhibit ribonucleotide reductase activity (19). Furthermore, in cell lines overproducing

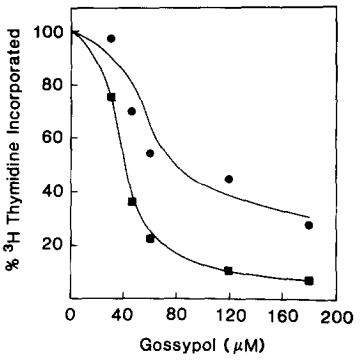


Figure 5: Percent of [^{3}H] thymidine incorporated into DNA in the presence of various concentrations of gossypol. Wild type mouse L cells (■) and ribonucleotide reductase overproducing mutant mouse L cells (●). The [^{3}H] thymidine labelling was performed as described in Materials and Methods.

ribonucleotide reductase activity DNA synthesis has been shown to be more resistant to inhibition by hydroxyurea than it is in wild type counterparts (13,19). The results of experiments showing the effect of gossypol on DNA synthesis in wild type mouse L cells and in the ribonucleotide reductase overproducing mutant cell line is shown in Figure 5. Clearly, DNA synthesis in the mutant cell line is more resistant to gossypol inhibition, a result which supports the view that ribonucleotide reductase is an important intracellular target of gossypol. In addition to this, preliminary results using rat myoblast cells selected for resistance to hydroxyurea (22) showed increases in ribonucleotide reductase activity and cross-resistance to the cytotoxic effects of gossypol (data not shown).

Attempts have been made to exploit the role of ribonucleotide reductase in the proliferation of neoplastic cells by developing antitumor drugs that selectively inhibit the reductase activity (eg. hydroxyurea and guanazole). Potent inhibition of ribonucleotide reductase activity by gossypol is consistent with the antiproliferative properties associated with this drug (4) and support the further investigation of gossypol as an antineoplastic agent. Furthermore, some of the effects exerted by this drug on spermatogenesis may be attributable to the inhibition of ribonucleotide reductase activity, necessary for duplication of the genetic material before recombination and reductive cell division. Since the prolonged use of antineoplastic agents in

man may be able to select for cells resistant to such agents (23), extensive treatment with gossypol could effectively promote the neoplastic state if cells resistant to the drug were inadvertantly selected in vivo by virtue of increased ribonucleotide reductase activity.

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REFERENCES

1. Chang, M.D., Gu, Z. and Saksina, S.S. (1980) Contraception 21, 461-469.
2. Colman, N., Gardner, A., and Herbert, V. (1979) Environ. Mutagen. 1, 315-320.
3. Majumdar, S.K., Ingraham, H.J. and Pyrmowicz, D.A. (1980) Cdn. J. Genet. Cytol. 24, 777-780.
4. Tso, W.W. (1984) Cancer Lett. 24, 257-261.
5. Tsui, Y.C., Creasy, M.R. and Hulten, M.A. (1983) J. Med. Genet. 20, 81-85.
6. Ye, W.S., Liang, J.C. and Hsu, T.C. (1983) In Vitro 19, 53-57.
7. Wang, Y and Rao, P.N. (1984) Cancer Res. 44, 35-38.
8. Thelander, L. and Reichard, P. (1979) Ann. Rev. Biochem. 48, 133-158.
9. Cory, J. G. and Sato, A. (1983) Mol. Cell. Biochem. 53, 257-266.
10. Wright, J.A. (1983) Pharmac. Ther. 22, 81-102.
11. Eriksson, S., Giraslund, A., Skog, S., Thelander, L. and Tribukait, B. (1984) J. Biol. Chem. 259, 11695-11700.
12. Graslund, A., Ehrenberg, A., and Thelander, L. (1982) J. Biol. Chem. 257, 5711-5715.
13. Akerblom, L., Ehrenberg, A., Graslund, A., Lankinen, H., Reichard, P. and Thelander, L. (1983) PNAS 78, 2159-2163.
14. Kuzik, B.A. and Wright, J.A. (1980) Biochem. Genet. 18, 311-331.
15. Steeper, J.R. and Stewart, C.D. (1970) Anal. Biochem. 34, 123-130.
16. Cory, J.G., Russell, F.A. and Mansell, M.M. (1973) Anal. Biochem. 55, 449-456.
17. Hards, R.G. and Wright, J.A. (1983) Cdn. J. Biochem. and Cell Biol. 61, 120-129.
18. Lewis, L.H. and Wright, J.A. (1974) Biochem. Biophys. Res. Comm. 60, 926-933.
19. Hards, R.G. and Wright, J.A. (1981) J. Cell. Phys. 106, 309-319.
20. Cory, J.G., Sato, A. and Lasater, L. (1981) Adv. Enz. Regulation 19, 139-149.
21. Thelander, L. and Graslund A. (1983) J. Biol. Chem. 258, 4063-4066.
22. Creasey, D.C. and Wright J.A. (1984) Biosci. Repts. 4, 299-309.
23. Goldie, J.H. and Coldman, A.J. (1984) Cancer Res. 44, 3643-3654.