[CANCER RESEARCH 44, 35–38, January 1984]

Effect of Gossypol on DNA Synthesis and Cell Cycle Progression of Mammalian Cells *in Vitro*[1]

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ABSTRACT

Gossypol, a yellow phenolic compound extracted from cotton plants that is being used as a male antifertility drug, has been found to have cytotoxic effects. The objective of the present study was to determine the cause of these cytotoxic effects in cultured cells and to ascertain whether cells in certain phases of the cell cycle were more sensitive to the drug than were cells in other phases. HeLa or Chinese hamster ovary cells were exposed to various concentrations of gossypol for varying periods of time, and the effects of the drug on the growth rate, cell cycle traverse, plating efficiency, and macromolecular synthesis were measured. The results of this study indicate that gossypol is a specific inhibitor of DNA synthesis; it has no effect on RNA and protein synthesis at a concentration of 10 μg/ml and hence has no effect on the cell cycle traverse of cells from G_2 to the beginning of S phase. In the presence of the drug, cells can enter S phase but fail to complete replication. The effects of the drug on growth rate and plating efficiency indicate that there is a threshold concentration at which gossypol becomes effective as a cytotoxic agent. Gossypol did not increase chromosome aberrations in the treated cells. Since gossypol irreversibly blocks cells in S phase, it could be useful as an antitumor drug.

INTRODUCTION

There is a renewed interest in gossypol because of its use as a male antifertility drug in China. This yellow phenolic compound, extracted from cotton seeds, stems, and roots, induces structural abnormalities in sperm of humans and males of other animal species when given p.o., effectively reducing the sperm count by 99.9% (3). Gossypol does not appear to be a mutagen according to the results of the Ames test (1) and the sperm head abnormality assay in mice (2).

Several reports deal with the effects of gossypol on somatic cells *in vitro*. Tsui *et al.* (8) observed no significant increase in chromosome aberrations and micronuclei or in the frequency of sister chromatid exchanges in phytohemagglutinin-stimulated human peripheral blood lymphocytes following incubation with gossypol, even at the highest concentration of 40 μg/ml. However, the mitotic index was reduced in gossypol-treated cultures. Using CHO[4] cells and human lymphocytes, Ye *et al.* (9) showed that gossypol treatment caused no increase in chromosome breakage or polypoidy but reduced the mitotic index, the rates of synthesis of DNA, RNA, and protein, and the percentage of viable cells as revealed by the dye exclusion method.

The objective of this study was to determine: (*a*) the cause for the reduction in mitotic index in gossypol-treated cultures; and (*b*) whether cells in certain phases of the cell cycle were more sensitive to the drug than were cells in other phases. Our data indicate that gossypol is a specific inhibitor of DNA synthesis with no adverse effects on RNA and protein synthesis at a moderately low concentration of 10 μg/ml.

MATERIALS AND METHODS

Cells and Cell Synchrony. HeLa and CHO cells were grown as monolayer cultures in a humidified, 5% CO_2 atmosphere at 37°, using McCoy's Medium 5A (Grand Island Biological Co., Grand Island, N. Y.), supplemented with glutamine (1%), antibiotics, and fetal calf serum (10%; KC Biologicals, Lenexa, Kans.). HeLa cells were synchronized in S phase by the excess dThd (2.5 mM) double-block method (6). Mitotic HeLa cells of 96 to 97% purity were obtained by the N_2O block method (5). Mitotic CHO cells were obtained by incubating cultures in medium containing Colcemid (0.25 μg/ml; Ciba Pharmaceuticals, Inc., Summit, N.J.).

Drugs. Gossypol of high purity was a generous gift from Dr. Pemmaraju N. Rao of Southwest Research Foundation, San Antonio, Texas. Gossypol was freshly prepared by dissolving it in dimethyl sulfoxide and then diluting with medium to give the desired concentration. The final concentration of dimethyl sulfoxide in the medium was ≤0.2% (v/v).

Cell Fusion. HeLa cells in exponential growth and those exposed to gossypol were pulse labeled with [^{3}H]dThd (1 μCi/ml; specific activity, 6.7 Ci/mmol) for 30 min, separately fused with mitotic HeLa cells by using UV-inactivated Sendai virus to induce premature chromosome condensation, and processed for chromosome spreads as described previously (7). The PCC were classified on the basis of their morphology into G_1-, S-, or G_2-PCC, and the percentages of cells residing in a particular phase of the cell cycle at the time of fusion were determined. After scoring for PCC, the slides were processed for autoradiography. The S-PCC were reexamined for the presence of label on them.

Drug Treatment and Plating Efficiency. HeLa or CHO cells in exponential growth, which were trypsinized and plated in a number of dishes 1 day before the experiment, were exposed to various concentrations of gossypol for various periods of time. At the end of the treatment, the medium containing the drug was removed, and cells were rinsed twice with drug-free medium, trypsinized, and plated in five 35-mm plastic dishes for each treatment at the rate of 100 cells/dish. After an incubation period of 8 to 10 days, the cell colonies were fixed, stained, and counted.

RESULTS

Effect on Growth Rate. Two concentrations of gossypol, 5 and 10 μg/ml, were evalulated. The effects of pulse teatment and continuous exposure to the drug on the growth rate of HeLa cells were studied. HeLa cells were plated in 60-mm plastic culture dishes; 24 hr later, the drug was added. Cell counts were taken at 24-hr intervals thereafter. Gossypol at 5 μg/ml for a 3- or 8-hr exposure and at 10 μg/ml for 3 hr has no significant

[1] Supported in part by USPHS Grants CA-27544 and CA-34783, awarded by the National Cancer Institute, Department of Health and Human Services and by Grant CH-205 from the American Cancer Society.

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[4] The abbreviations used are: CHO, Chinese hamster ovary; dThd, thymidine; PCC, prematurely condensed chromosomes.

Received July 27, 1983; accepted September 14, 1983.

effect on the growth rate (Chart 1). The continuous presence of drug in culture medium, even at a low dose of 5 μg/ml, caused a plateau in growth rate after a slight increase in cell number. A drug concentration of 10 μg/ml for 8 hr totally inhibited growth. The continuous presence of the drug in the medium at 10 μg/ml resulted in cell lysis and a decrease in cell number with time.

Effect on Plating Efficiency. HeLa cells were exposed to 5, 7.5, 10, or 20 μg/ml for either 3 or 8 hr and then plated for colonies. Once again, we found that gossypol at 5 μg/ml for 3 or 8 hr and at 10 μg/ml for 3 hr had no measurable effect on the plating efficiency (Table 1). Virtually no colonies grew when HeLa cells were treated with the drug at 10 μg/ml for 8 hr or at 20 μg/ml for 3 hr. We did not observe a gradual decline in plating efficiency with increase in dose. The all-or-none effect seen here suggests that there is a threshold concentration at which the drug becomes cytotoxic. The critical concentration can be achieved by exposing cells to either higher drug concentrations for shorter times or to lower concentrations for longer times.

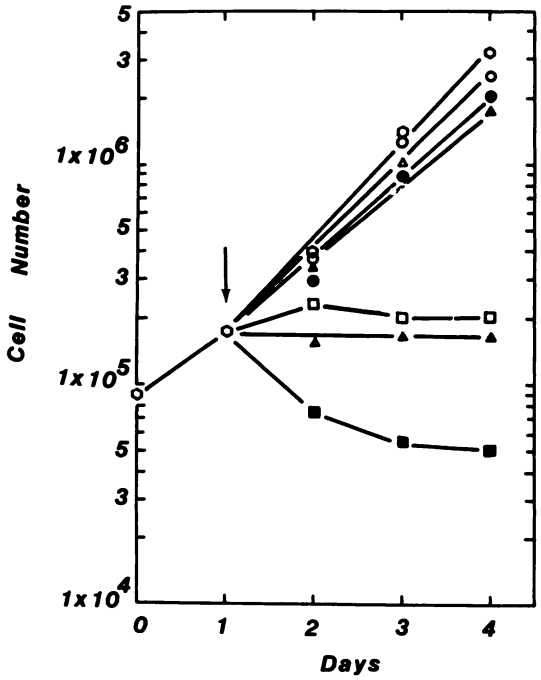

Chart 1. Effect of gossypol on the growth rate of HeLa cells. Monolayer cultures in exponential growth were trypsinized and cells were plated 1 day before gossypol was added to the dishes at various concentrations, and cells were exposed to the drug for varying periods of time. Cell counts represent an average of 2 samples for each *time point*; data are the average of 2 sets of experiments. ○, untreated control random culture; cells treated with gossypol (5 μg/ml) for 3 hr (○), 8 hr (△), and continuously (□) throughout the duration of the experiment. Cells treated with gossypol (10 μg/ml) for 3 hr (●), 8 hr (▲), and continuously (■). *Arrow*, time of addition of gossypol to the culture dishes.

Table 1

Effect of gossypol on the plating efficiency of HeLa cells

Dose	3 hr treatment		8 hr treatment	
	Actual no. of colonies	Relative plating efficiency	Actual no. of colonies	Relative plating efficiency
Control	88	100	81	100
Gossypol (5 μg/ml)	92	104	86	106
Gossypol (7.5 μg/ml)	92	104	ND[a]	ND
Gossypol (10 μg/ml)	93	105	0	0
Gossypol (20 μg/ml)	0	0	0	0

[a] ND, not done.

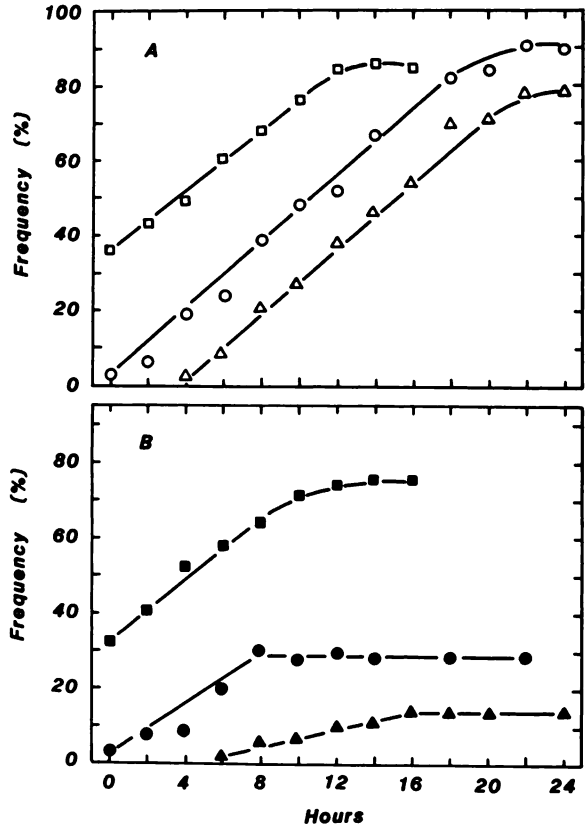

Chart 2. Effect of gossypol on HeLa cell cycle traverse. Two sets of 14 each of 35-mm dishes were plated with HeLa cells in exponential growth. Twenty-four hr after trypsinization and plating, old medium in all the dishes was replaced with fresh medium containing Colcemid (0.05 μg/ml) and [^{3}H]dThd (0.05 μCi/ml; specific activity, 6.7 Ci/mmol). Gossypol (10 μg/ml) was added to one set of dishes, while the other set served as control. Cell samples were taken at regular intervals by trypsinizing one of the dishes to determine the labeling index (□, ■), total (labeled plus unlabeled) mitotic index (○, ●), and labeled mitotic index (△, ▲). *A*, control; *B*, gossypol-treated cultures. Note that the rate of increase in labeling index, *i.e.*, G_1 to S-phase transition, is identical in both the control and treated cultures. The total mitotic index in the treated cells leveled off after an initial increase. The slope indicates the rate of entry of G_2 cells and some S-phase cells into mitosis. The labeled mitotic index curve, which indicates the rate of entry of S-phase cells into mitosis, leveled off after a very small increase. This suggests that most of the cells in S-phase at the time of the addition of the drug failed to reach mitosis.

Effect of Gossypol on Cell Cycle Progression. To determine the cell cycle phase specificity of the drug, we performed cell cycle analysis according to the labeled-mitosis accumulation method of Puck and Steffen (4). The continuous presence of gossypol (10 μg/ml) in the growth medium did not inhibit the progression of G_1 cells into S phase or G_2 cells into mitosis (Chart 2). Only a small proportion of those cells in S phase at the time of addition of the drug entered mitosis (Chart 2*B*). Most of the S phase and the G_1 cells that entered S phase subsequent to the addition of the drug failed to reach mitosis, as indicated by the plateau in the total and labeled mitotic index curves (compare Chart 2*B* with Chart 2*A*). These data indicate that cells can enter S phase in the presence of the drug but that most of them are not able to complete replication and proceed to mitosis.

These results were further confirmed by using HeLa cells synchronized in various phases of the cell cycle. Gossypol had no effect on the progression of synchronized mitotic cells into G_1, G_1 cells into S phase (Chart3*A*), and G_2 cells into mitosis (Chart 3*C*). When gossypol was added to cells in early S phase, *i.e.*, 1 hr after reversal of the second dThd block, there was a

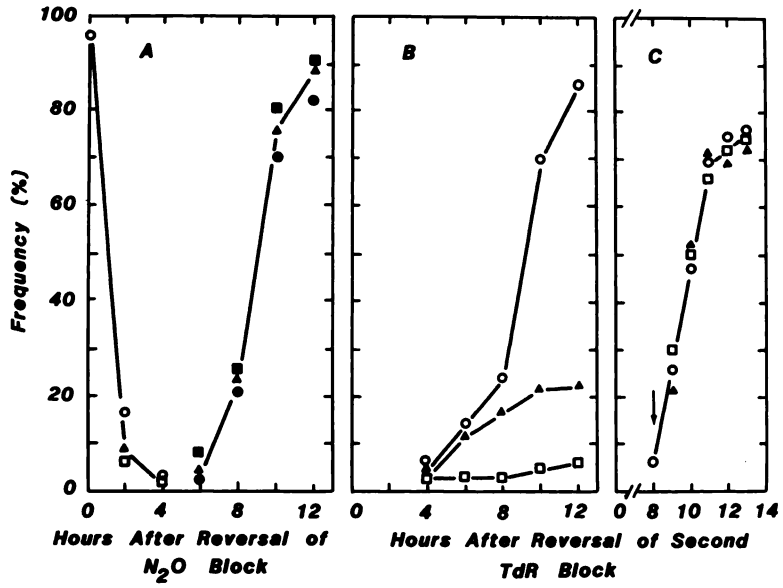

Chart 3. Effect of gossypol on the cell cycle traverse of synchronized HeLa cells. ○, △, □, mitotic index; ●, ▲, ■, labeling index. Cells were continuously exposed to gossypol (10 μg/ml) (△), and gossypol (20 μg/ml) (□), throughout the course of the experiment. ○, control. *A*, effect on the progression of synchronized mitotic cells into S phase. [^{3}H]dThd (*TdR*) (0.1 μCi/ml) and various concentrations (0, 10, and 20 μg/ml) of gossypol were added to the dishes at t = 0 hr. Colcemid (0.05 μg/ml) was added at 4 hr after reversal of N_2O block. *B*, effect on the progression of synchronized S cells into mitosis. Colcemid (0.05 μg/ml) and various concentrations of gossypol were added at t = 0 hr. *C*, effect on the progression of G_2 cells into mitosis. Colcemid (0.05 μg/ml) and various concentrations of gossypol were added at 8 hr after the reversal of the second [^{3}H]dThd block (*arrow*).

Table 2

Effect of gossypol treatment on the cell cycle traverse of CHO cells

Treatment	Frequency (%) PCC in		
	G_1	S	G_2
Control (no drug)	50	38	12
Gossypol (5 μg)	2	96	3
Gossypol (10 μg)	1	98	1

dose-dependent inhibition in their progression to mitosis (Chart 3*B*). The mitotic accumulation in the presence of Colcemid was abut 20% at a drug concentration of 10 μg/ml and 6% at the 20-μg/ml dose.

S-Phase Arrest Induced by Gossypol. To determine whether the gossypol-treated cells were blocked in S phase or G_2, a random population of CHO cells were treated with gossypol (10 μg/ml) for 48 hr, pulse labeled with [^{3}H]dThd (1.0 μCi/ml) for 30 min, and then fused with synchronized mitotic CHO cells to induce premature chromosome condensation. The PCC of the control and gossypol-treated cultures were classified as G_1-, S-, or G_2-PCC based on their morphology as described by Rao *et al.* (7). Almost all of the cells in the gossypol-treated cultures were arrested in S phase as indicated by their characteristic pulverized appearance (Table 2). Very few of them incorporated [^{3}H]dThd, suggesting that DNA synthesis was almost totally inhibited.

Lack of Effect of Gossypol on Cell Cycle Traverse through G_2, Mitosis, and G_1. To confirm that gossypol specifically blocked cells in S phase and had no effect on the traverse of cells through the rest of the cell cycle, HeLa cells were synchronized in S phase by the excess dThd double-block method. Eight hr after the reversal of the second dThd block, when most of the cells had completed DNA replication and entered G_2, we added gossypol (10 μg/ml) to the monolayer culture and placed the dishes in a N_2O chamber (80 psi) to synchronize cells in mitosis. At 8 hr of incubation in the N_2O chamber, the rounded and loosely attached mitotic cells were collected by selective detachment and plated in 35-mm dishes in fresh medium containing gossypol (10 μg/ml) and [^{3}H]dThd (0.1 μCi/ml; 6.7 Ci/mmol). Cell samples were taken at 2-hr intervals, fixed, processed, and scored for mitotic and labeling indices. The mitotic cells collected in the presence of gossypol divided and traversed G_1 and entered S phase almost at the same rate as those from the untreated control culture (data not shown). These data clearly indicate that the cytotoxic effects of gossypol are confined to the S period of the cell cycle.

Effect of Gossypol on Macromolecular Synthesis. The effects of gossypol (10 μg/ml) on the rates of synthesis of DNA, RNA, and protein were measured by pulse labeling the control and drug-treated cultures with appropriate ^{3}H-labeled precursors at different times after the addition of the drug. The rates of RNA and protein synthesis were unaffected by the presence of gossypol in the medium (Chart 4). In contrast, the rate of DNA synthesis decreased to approximately 50 and 25% of the control by 1 and 8 hr, respectively. It dropped to zero by 24 hr of treatment (data not shown).

Effect of Gossypol on Chromosome Structure. HeLa cells exposed to gossypol (5 and 10 μg/ml) for 24 hr were incubated with Colcemid (0.05 μg/ml) for 4 hr, and chromosome spreads were prepared. The chromosome spreads of the control and gossypol-treated cultures were scored for chromosome aberrations, *i.e.*, gaps, breaks, and exchanges. We observed no significant difference in the frequency of these aberrations between the control and the drug-treated cells (data not shown).

DISCUSSION

The results of this study indicate that: (*a*) gossypol is a specific inhibitor of DNA synthesis and has no effect on RNA and protein synthesis at a concentration of 10 μg/ml; (*b*) there is a threshold concentration at which the drug becomes effective as a cytotoxic

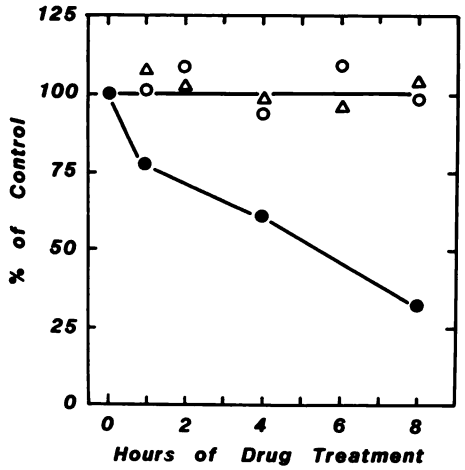

Chart 4. Effect of gossypol on DNA, RNA, and protein synthesis in HeLa cells. Two sets of 18 each of 35-mm dishes were plated with equal numbers of cells (approximately, 1×10^5 cells/dish) 1 day before the experiment. The experiment was begun by adding gossypol (10 μg/ml) to one set of dishes, while the other set served as control. Colcemid (0.05 μ/ml) was added to both sets of dishes to block cell division and to hold the cell number constant. At various times after the addition of gossypol, dishes from the control and drug-treated cultures were pulse labeled for 30 min with [^{3}H]dThd (1.0 μCi/ml), [^{3}H]deoxyuridine (1.0 μCi/ml), or [^{3}H]leucine (2 μCi/ml) and processed for scintillation counting to measure the incorporation of label into DNA (●), RNA (○), and protein (△), respectively.

agent; and (c) the drug does not damage chromosomes in the treated cells. Besides HeLa cells, we have repeated these experiments with CHO cells and human diploid fibroblasts with similar results.

In an earlier study, Ye *et al.* (9) reported a significant inhibition of all macromolecular synthesis in CHO cells treated with gossypol when they used much higher doses of the drug than we did; however, at the lower concentration of 10 μg/ml, their data are similar to ours. RNA and protein synthesis are known to be essential for the progression of G_1 cells into S phase and G_2 cells into mitosis. Gossypol at a concentration of 10 μg/ml had no effect on the cell cycle traverse of cells from G_2 to the beginning of S phase. The cytotoxic effects of gossypol appear to be due to the irreversible inhibition of DNA synthesis, which is manifest only when a threshold concentration of the drug within the cells is achieved either by higher concentrations or by longer exposures. Although the mechanism of this inhibition remains to be elucidated, the data suggest that gossypol may be useful as a potential antitumor drug. At present, it is being evaluated for its antitumor effects in experimental animal systems.

As in earlier studies by others, we observed no increase in the incidence of chromosome aberrations in HeLa cells treated with gossypol for prolonged periods. In the light of these and other published reports, it is clear that gossypol is neither a mutagen nor a clastogen.

The mechanism of the specific actions of gossypol on spermatogenesis is not yet clearly understood. Since the side effects of gossypol, when administered p.o. as a male contraceptive, are minor, it is likely that threshold concentration of the drug within the cells may not have been achieved to cause any cytotoxic effects.

ACKNOWLEDGMENTS

We thank Josephine Neicheril for her assistance in the preparation of this manuscript.

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