Biochemical Pharmacology, Vol. 46, No. 2, pp. 251–255, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

ANTI-HIV-1 ACTIVITY AND CELLULAR PHARMACOLOGY OF VARIOUS ANALOGS OF GOSSYPOL

TAI-SHUN LIN,*† RAYMOND F. SCHINAZI,‡ JULIANG ZHU,*† EVELYN BIRKS,*
ROCCO CARBONE,† YIKANG SI,§ KEMEI WU,§ LIANG HUANG§ and
WILLIAM H. PRUSOFF*‖

*Department of Pharmacology and †Comprehensive Cancer Center,
Yale University School of Medicine, New Haven, CT 06510;
‡Veterans Affairs Medical Center (Atlanta), Decatur, GA 30033, and Department of Pediatrics,
Emory University School of Medicine, Atlanta, GA 30322; and §Institute of Materia Medica,
Chinese Academy of Medical Sciences, Beijing, China

(*Received* 19 *February* 1993; *accepted* 19 *March* 1993)

Abstract—We previously reported that the racemic mixture and both enantiomers of gossypol inhibit the replication of human immunodeficiency virus-type 1 (HIV-1) (Lin *et al.*, *Antimicrob Agents Chemother* **33**: 2149–2151, 1989). The present study evaluates the activities of a variety of analogs of gossypol as well as a few non-gossypol analogs. Compounds **2**, **3**, **10**, and **13** were slightly more inhibitory than (−)-gossypol to the replication of HIV-1 in cell culture. Compounds **4** and **8** were cytotoxic to human peripheral blood monocyte (PBM) cells, and compounds **2** and **3** were cytotoxic to Vero cells but not PBM cells. The effects of the two enantiomers of gossypol on the cell volume and migration of H9 cells through the cell cycle were evaluated during 72 hr of incubation. The (−)-enantiomer of gossypol was more toxic to H9 cells than the (+)-enantiomer of gossypol as evidenced by cell destruction. Prior to cell destruction, there appeared to be no significant effect on cell cycle distribution with either enantiomer.

Gossypol is a natural product extracted from the cottonseed, which has been studied extensively as a potential male contraceptive and has been well reviewed by Qian and Wang [1]. When isolated from cottonseed, gossypol is a racemic mixture. The enantiomeric forms are due to atropisomerism, i.e. restricted rotation of the two naphthyl rings about the interlinking C—C bond (Fig. 1). The two enantiomers have been resolved by several groups [2–4]. Each enantiomer can exist in solution in three tautomeric forms. Gossypol has antiparasitic activities [5, 6] and antiviral activities against enveloped viruses such as herpes simplex virus type 2 (HSV-2) and para-influenza virus type 3, but not against non-enveloped poliovirus [7–9]. Polsky *et al.* [10] incubated the HTLV-III B strain of human immunodeficiency virus with gossypol in a cell-free medium and found that gossypol prevents recovery of viable viruses when subsequently incubated with H9-T cells. The racemic mixture (±) and the two pure enantiomers were evaluated against human immunodeficiency virus-type 1 (HIV-1¶) by Lin *et al.* [11]. We found the (−)-enantiomer of gossypol to be very inhibitory (EC_{50} = 1.0 to 5.0 μM), whereas the (+)-enantiomer was much less active (EC_{50} = 50 to 100 μM). The cytotoxicity (IC_{50}) for the (−)-enantiomer to uninfected human peripheral blood mononuclear (PBM) cells was > 100 μM, and 52 μM for the (+)-enantiomer.

Since toxicity of gossypol may be a result of a Schiff base type of reaction between the aldehyde group and cellular proteins, Radloff and colleagues [9] evaluated a series of gossylic nitrile-1,1′-diesters of which the diacetate and divalerate inhibited HSV-2 replication in Vero cells that were infected with the virus before administration of the drug. They found that modification of the aldehyde functional groups on gossypol lowers the toxicity of the drug, but does not abolish its antiviral properties. Antiviral activity was noted with gossypol concentrations as low as 0.5 μM. Royer *et al.* [12, 13] also found such modifications to be less toxic than the parent compound. Various analogs of gossypol have been synthesized and reported in the literature [14–16]. The present report presents the antiviral activity against HIV-1 of several gossypol derivatives, their cytotoxicity to PBM and Vero cells, and their effects on the cell volume and cell cycle of H9 cells.

MATERIALS AND METHODS

Assay for antiviral activity. For studies with PBM cells, 3-day-old mitogen-stimulated cells (10^6/mL) were infected with HIV-1 (LAV) at a concentration of about 100 $TCID_{50}$ (50% tissue culture infective doses per mL equivalent to about 60,000 dpm of reverse transcriptase activity per mL) and cultured

‖ Corresponding author: Dr. William H. Prusoff, Department of Pharmacology, Yale University School of Medicine, 333 Cedar St., P.O. Box 3333, New Haven, CT 06510-8066. Tel. (203) 785-4378; FAX (203) 785-7670.

¶ Abbreviations: HIV-1, human immunodeficiency virus-type 1; PBM, peripheral blood monocyte; EC_{50}, concentration required to reduce the virus yield by 50%; IC_{50}, concentration required to reduce the cell number by 50%; $TCID_{50}$, dose of HIV-1 required to inhibit 50% of cells; and DMSO, dimethyl sulfoxide.

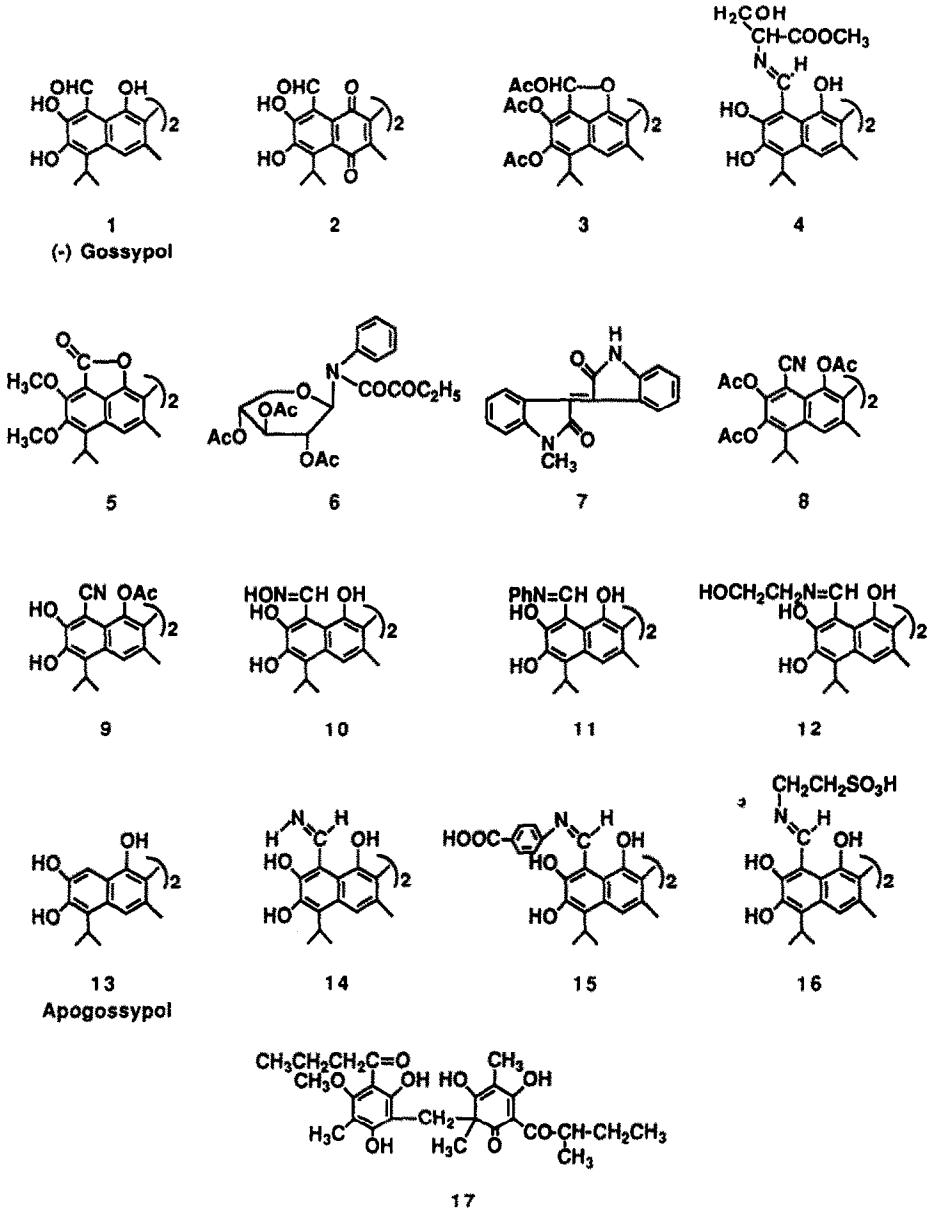

Fig. 1. Structures of various analogs of gossypol and a few related non-gossypol structures.

in the presence and absence of various concentrations of (−)-gossypol and gossypol derivatives. Six days after infection, the supernatant was clarified and the virus was pelleted at 147,000 *g*. The reverse transcriptase activity in the disrupted virus was determined. The methods for cultivating the PBM cells, harvesting the virus, and determining the reverse transcriptase activity have been reported previously [17]. The concentrations required to reduce virus yield by 50% (EC_{50}) were determined by the median effect method [18].

Assays for cytotoxicity. Cytotoxicity studies were determined with uninfected PBM and Vero cells as previously reported [17]. The concentration required to reduce the cell number relative to control by 50% (IC_{50}) was determined.

Effect on DNA synthesis. Studies were performed on the effects of several of the compounds on the utilization of [^{3}H]thymidine by PBM cells as described previously [17].

Effects of the enantiomers of gossypol on relative cell volume and the cell cycle progression of H9 cells. H9 cells were grown in RPMI 1640 medium supplemented with 2% heat-inactivated fetal bovine serum and 1% each of 200 mM glutathione, 1 M HEPES (pH 7.3), 100X HL-1 supplement (Ventrex Laboratories) and heat-inactivated newborn calf serum.

(−)-Gossypol and the various analogs of gossypol were each dissolved in dimethyl sulfoxide (DMSO, 49.3 mM) and diluted with culture medium to a concentration of 700 μM for preparation of the stock

Table 1. Effects of (−)-gossypol and various analogs of gossypol on the replication of HIV-1 in PBM cells and cytotoxicity of PBM and Vero cells

Compound	Inhibition of HIV-1 in PBM cells EC_{50} (μM)	Cytotoxicity in PBM cells IC_{50} (μM)	Inhibition of [^{3}H]dThd utilization in PBM cells IC_{50} (μM)	Cytotoxicity in Vero cells IC_{50} (μM)
1	1.7	>100	4.6	5.2
2	0.86	>100	1.7	0.87
3	0.93	>100	2.3	4.1
4	2.2	35.0	14.4	1.2
5	>100	>100	ND*	>100
6	9.3	>100	1.0	>100
7	>100	>100	ND	>100
8	5.0	8.7	2.7	2.7
9	26.5	>100	>100	>100
10	0.41	>100	3.2	7.5
11	91.5	>100	>100	ND
12	6.0	>100	1.2	ND
13	0.82	>100	4.4	77.4
14	3.4	>100	2.1	6.6
15	47.8	>100	6.2	13.5
16	17.3	>100	16.0	37.5
17	3.92	>100	2.0	≥1.0
AZT (control)	0.004	>100	ND	29.0

* ND = not determined.

solution. Gossypol solutions were diluted further with culture medium containing 1.42% DMSO such that when 4 mL of solution was added to 101 mL of H9 cells (6.3×10^4/mL) the following concentrations in 0.054% DMSO were achieved: control, no gossypol; (−)-gossypol, 2 and 5 μM; (+)-gossypol, 10 and 25 μM.

After 0, 3, 6, 12, 24, 48, and 72 hr of incubation at 37° with 5% CO_2, 1 mL was removed for cell counting, and a volume containing 10^6 cells was used for flow cytometry. The cells for analysis by flow cytometry were centrifuged, and the cell pellets were gently washed twice with ice-cold phosphate-buffered saline (PBS). They were then suspended with cold PBS (2 mL). Three portions of cold 95% EtOH (2 mL) were added with gentle mixing between additions giving approximately a 70% EtOH suspension of fixed cells; these fixed cells when stored at 4° were quite stable until all the samples were processed for analysis of their cell-cycle distribution. After a minimum of 1-hr fixation, the cells were resuspended in 1 mg/mL RNAse (Sigma) for 30 min at 37°, and then stained with 0.05 mg/mL propidium iodide (Sigma) for 1 hr on ice.

Flow cytometric analysis was performed with a FACS IV flow cytometer (Becton-Dickinson, San Jose, CA). The cells were excited at 488 nm and the emission was collected above 590 nm. A minimum of 20,000 cells was analyzed for each sample. Cell cycle analysis was performed according to the mathematical model of Jett [19].

RESULTS AND DISCUSSION

The effects of gossypol and various derivatives on the replication of HIV-1 in human PBM cells are given in Table 1. Compounds **2**, **3**, **10**, and **13** were slightly more inhibitory than (−)-gossypol (compound **1**), ranging from a factor of 1.8 to 4.2. With the exception of compounds **4** and **8**, none of the other compounds demonstrated any apparent cytotoxicity to human PBM cells. Although nontoxic to PBM cells, compounds **2**, **3**, **4**, **10**, **14**, and **17** were markedly toxic to Vero cells. When the uptake of [^{3}H]thymidine was used as a marker for toxicity, compounds **1**, **2**, **3**, **6**, **8**, **10**, **12**, **13**, **14**, **15**, and **17** exhibited significant toxicity (IC_{50}: < 10 μM).

Effect on cell volume and cell cycle of H9 cells. The effect of (−)-gossypol on the cell volume and migration through the cell cycle is shown in Fig. 2 using two levels of (−)-gossypol (2 and 5 μM) for 0–72 hr of incubation. A slight decrease in cell volume of questionable significance was observed after 12 hr of incubation with 2 μM (−)-gossypol, which increased slightly upon continued incubation up to 72 hr. There was no marked effect on the distribution in the cell cycle.

At 5 μM (−)-gossypol, a small decrease in cell volume could be detected and an amount similar to that produced by 2 μM (−)-gossypol. However, this effect on cell volume was more apparent as incubation continued. By 24 hr, a small decrease in the number of cells in M/G_2 was observed, and cellular degradation became more marked as incubation continued up to 72 hr.

Studies with H9 cells indicated that the (−)-enantiomer of gossypol was more cytotoxic than (+)-gossypol (Figs. 2–4). Two levels of (+)-gossypol (10 and 25 μM) were evaluated. The lower concentration (10 μM) produced a very slight decrease in cell volume after 24 hr of incubation, but only after 72 hr of incubation was there evident formation of cellular debris. However, there was no

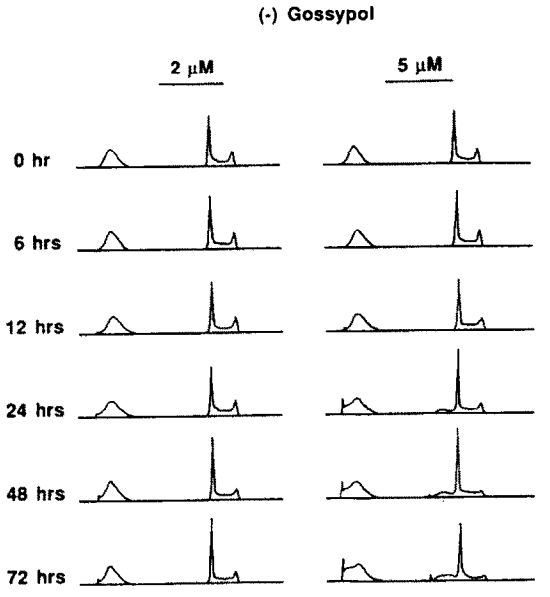

Fig. 2. Changes in cell volume and cell-cycle distribution of H9 cells during continuous cell growth in the presence of 2 or 5 μM (−)-gossypol.

apparent effect on the cell cycle distribution of H9 cells.

(+)-Gossypol at a 25 μM concentration was detectably toxic to H9 cells as early as after 6 hr of incubation. A small decrease in cell volume was observed, which was more marked after 12 hr of incubation, with almost total destruction by 24 hr of incubation. The destructive effect was reflected in the cell cycle distribution as early as 12 hr of incubation.

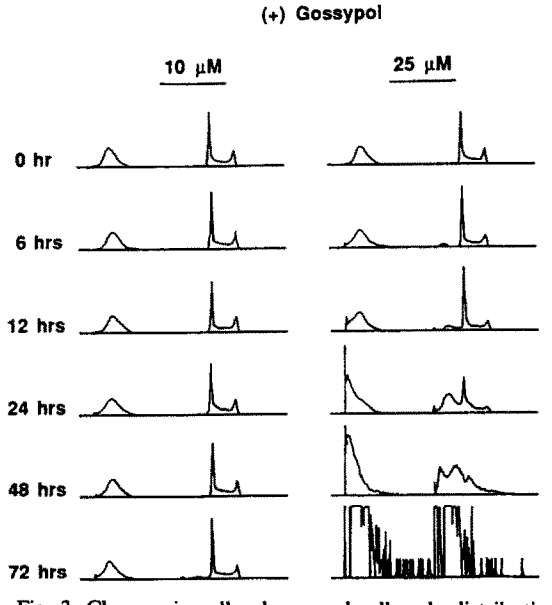

Fig. 3. Changes in cell volume and cell-cycle distribution of H9 cells during continuous cell growth in the presence of 10 or 25 μM (+)-gossypol.

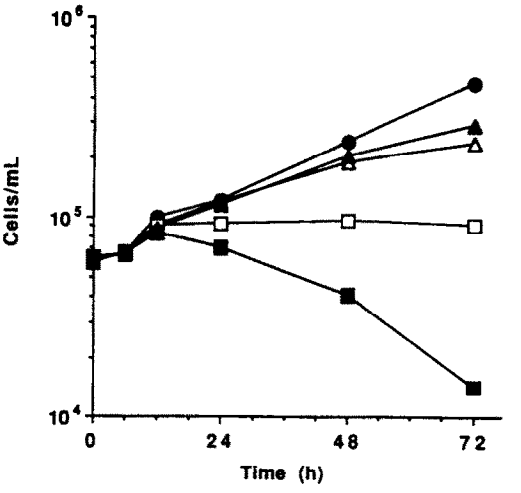

Fig. 4. Effect on replication of H9 cells in medium supplemented with two levels of (−)-gossypol, 2 μM (△) and 5 μM (□); with two levels of (+)-gossypol, 10 μM (▲) and 25 μM (■); and no gossypol (●).

Wang and Rao [20] evaluated the effect of gossypol on the cell cycle progression of HeLa and Chinese hamster cells, and found that at a concentration of 10 μg/mL (19.3 μM) gossypol blocked synchronized HeLa cells in S phase but had no effect on cells progressing into G_1, G_1 cells into S phase, and G_2 cells into mitosis. The difference in results from our findings may be related to different cell lines that appear to be more susceptible to inhibition by gossypol, or their use of a racemic gossypol rather than the enantiomers.

Lin *et al.* [11] reported that (−)-gossypol is more inhibitory than (+)-gossypol to the replication of HIV-1 in human PBM cells but less cytotoxic; however, similar studies in H9 cells indicated that (−)-gossypol, although more inhibitory to the replication of HIV-1, is more cytotoxic to these cells than (+)-gossypol at a 50 μM concentration, but not at 5 μM. Studies by Joseph *et al.* [21] on the cytotoxicity to BCL-D1 cells, a human diploid strain of fibroblasts, found (−)-gossypol to be about 10-fold more toxic than (+)-gossypol and racemic gossypol to be 2.5-fold more toxic. In the present study with H9 cells, 5 μM (−)-gossypol was more inhibitory than 10 μM (+)-gossypol in regard to their relative effect on cell volume, cell cycle, and replication of H9 cells.

We previously found that gossypol inhibited the replication of HIV-1, and using this as a lead compound we examined various analogs of gossypol. Although differences in inhibition and cytotoxicity were found, none was of the magnitude to justify further study.

Acknowledgements—This research was supported by Public Health Service Grants CA-05262, CA-44094, and CA-08341 from the National Cancer Institute and AI-27196, AI-25899 and AI-29430 from the National Institute of Allergy and Infectious Diseases and by the Veterans Affairs. We also acknowledge support for the cytometry studies from the Yale Comprehensive Cancer Center Flow

Cytometry Core Facility, U.S. Public Health Service Grant CA-16359 from the National Cancer Institute. Special thanks to Diane Mozdziesz for preparation of the figures, and to Rosemary Kirck for preparation of the manuscript.

REFERENCES

1. Qian S-Z and Wang Z-G, Gossypol: A potential antifertility agent for males. *Annu Rev Pharmacol Toxicol* **24**: 329–360, 1984.
2. Sampeth DS and Balaram P, Resolution of racemic gossypol and interaction of individual enantiomers with serum albumins and model peptides. *Biochim Biophys Acta* **882**: 183–186, 1986.
3. Si YK, Zhou J and Huang L, (−)-Gossypol from resolution of racemic gossypol. *Kue-Xue Tong Bao* **28**: 640, 1983 (English Edn **28**: 1574, 1983).
4. Matlin SA and Zhou RJ, Resolution of gossypol: Analytical and preparative HPLC. *J High Resolut Chromatogr Commun* **7**: 629–631, 1984.
5. Montamat EE, Burgos C, Gerez de Burgos NM, Rovail LE, Blanco A and Segura EL, Inhibitory action of gossypol on enzymes and growth of *Trypanosoma cruzi*. *Science* **218**: 288–289, 1982.
6. Vander Jagt DL, Boack BR, Campos NM, Hunsaker LA and Royer RE, A derivative of gossypol retains antimalarial activity. *IRCS Med Sci* **12**: 845–846, 1984.
7. Dorsett PH, Kerstine EE and Powers LJ, Antiviral activity of gossypol and apogossypol. *J Pharm Sci* **64**: 1073–1075, 1975.
8. Wichmann K, Vaheri A and Luukkainen T, Inhibiting herpes simplex virus type 2 infection in human epithelial cells by gossypol, a potent spermicidal and contraceptive agent. *Am J Obstet Gynecol* **142**: 593–594, 1982.
9. Radlof RJ, Deck LM, Royer RE and Vander Jagt DL, Antiviral activities of gossypol and its derivatives against herpes simplex virus type II. *Pharmacol Res Commun* **18**: 1063–1073, 1986.
10. Polsky B, Gold JWM, Baron PA, Ueno H, Segal SJ, Halstead SB and Armstrong D, Inactivation of human immunodeficiency virus (HIV) by gossypol (GP). *Clin Res* **35**: 487A, 1987.
11. Lin T-S, Schinazi R, Griffith BP, August EM, Eriksson BFH, Zheng D-K, Huang L and Prusoff WH, Selective inhibition of human immunodeficiency virus type 1 replication by the (−) but not the (+) enantiomer of gossypol. *Antimicrob Agents Chemother* **33**: 2149–2151, 1989.
12. Royer RE, Deck LM, Campos NM, Hunsaker LA and Vander Jagt DL, Biologically active derivatives of gossypol: Synthesis and antimalarial activities of peri-acylated gossylic nitriles. *J Med Chem* **29**: 1799–1801, 1986.
13. Royer RE, Mills RG, Deck LM, Mertz GJ and Vander Jagt DL, Inhibition of human immunodeficiency virus type I replication by derivatives of gossypol. *Pharmacol Res* **24**: 407–412, 1991.
14. Petrov OS, Ognyanov VI, Tiholov EP and Mollov NM, Synthesis of gossypol analogues. *Helv Chim Acta* **72**: 353–360, 1989.
15. Kunesch N, Barbier MP, Dalkafouki A and Poisson JE, Preparation de derives de la fonction aldehyde du gossypol. *Ann Pharm Fr* **48**: 1–6, 1990.
16. Deck LM, Vander Jagt DL and Royer RE, Gossypol and derivatives: A new class of aldose reductase inhibitors. *J Med Chem* **34**: 3301–3305, 1991.
17. Schinazi RF, Sommadossi J-P, Saalmann V, Cannon DL, Xie M-W, Hart GC, Smith GA and Hahn EF, Activity of 3′-azido-3′-deoxythymidine nucleotide dimers in primary lymphocytes infected with human immunodeficiency virus type 1. *Antimicrob Agents Chemother* **34**: 1061–1067, 1990.
18. Schinazi RF, Chou IC, Scott RJ, Yao X and Nahmias AJ, Delayed treatment with combinations of antiviral drugs in mice infected with herpes simplex virus and application of the median effect method of analysis. *Antimicrob Agents Chemother* **30**: 491–498, 1986.
19. Jett JH, Mathematical analysis of DNA histograms from asynchronous and synchronous cell populations. In: *Pulse Cytophotometry* (Ed. Lutz D), pp. 93–102. European Press, Belgium, 1978.
20. Wang Y-C and Rao PN, Effect of gossypol on DNA synthesis and cell cycle progression of mammalian cells *in vitro*. *Cancer Res* **44**: 35–38, 1984.
21. Joseph AEA, Matlin SA and Knox P, Cytotoxicity of enantiomers of gossypol. *Br J Cancer* **54**: 511–513, 1986.