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Biochemical Correlates of the Antitumor and Antimitochondrial Properties of Gossypol Enantiomers

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SUMMARY

Racemic gossypol has been shown to have antitumor properties that may be due to its ability to uncouple tumor mitochondria or to its inhibitory effects on a variety of nonmitochondrial enzymes. We have studied the antimitochondrial and enzyme-inhibiting properties of gossypol in human carcinoma cell lines of breast (MCF-7, T47-D), ovarian (OVCAR-3) colon (HCT-8), and pancreatic (MiaPaCa) origin by comparing the effects of its purified (+)- and (−)-enantiomers. (−)-Gossypol shows up to 10-fold greater antiproliferative activity than (+)-gossypol in the cancer cell lines and in normal hematopoietic stem cells grown *in vitro*, with IC_{50} values ranging from 1.5 to 4.0 μM for the cancer cells and from 10 to 20 μM for the human marrow stem cells. As well, multidrug-resistant MCF/Adr cells appear more resistant to (−)-gossypol than their parental cell line. Electron microscopy indicates that the earliest ultrastructural change in tumor cells exposed to a cytotoxic (10 μM) concentration of (−)-gossypol is the selective destruction of their mitochondria. Consistent with this observation, ^{31}P magnetic resonance spectroscopy detects pronounced changes in tumor cell high energy phosphate metabolism within 24 hr of (−)-gossypol treatment, manifest by 1.6- to >50-fold differential reductions in the intracellular ratios of ATP/P_i, relative to (+)-gossypol-treated cell lines; the magnitude of these antimitochondrial effects correlates with the antiproliferative activity of (−)-gossypol. Northern blot RNA analyses suggest that treatment with a 5–10 μM dose of (−)-gossypol induces a transient increase in the expression of heat shock gene products, particularly *hsp*-70 transcripts. The mean 5-fold increase in (−)-gossypol-induced *hsp*-70 mRNA appears coincident with a comparable heat-stimulated increase in transcript levels, as compared with control or (+)-gossypol-treated cells. The enzyme-inhibiting properties of gossypol enantiomers were compared in cell-free assays measuring glutathione-*S*-transferase-α, -μ, and π activities, calmodulin stimulation of cyclic nucleotide phosphodiesterase, and protein kinase C activity. Both enantiomers are near equivalent antagonists of calmodulin stimulation and protein kinase C activity, exceeding the potency of known inhibitors such as phenothiazines by as much as 50-fold. In contrast, (−)-gossypol is a 3-fold more potent inhibitor of glutathione-*S*-transferase-α and -π isozyme activity, resulting in IC_{50} values of 1.6 and 7.0 μM, respectively, for these two isozymes. Because of the enhanced resistance of MCF/Adr cells to (−)-gossypol, which may be related to their increased glutathione-*S*-transferase and protein kinase C content, (−)-gossypol should be evaluated for its potential to modify the cytotoxic resistance of human carcinoma cells to other chemotherapeutic agents. Furthermore, the above newly described (+)- and (−)-gossypol effects may be useful in directing structure-function studies using chiral-specific gossypol derivatives, in order to develop more selective and potent antimitochondrial chemotherapeutic agents.

Racemic gossypol [1,1′,6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl-(2,2′-binaphthalene)-8,8′-dicarboxyaldehyde] is a phenolic extract from cotton seeds first used in China as a male oral contraceptive and later found to have *in vitro* and *in vivo* antitumor properties (1, 2). As with certain other polycyclic lipophilic agents, gossypol appears to sequester within the mitochondria of tumor cells, where it uncouples oxidative phosphorylation from electron transport and reduces intracellular ATP, a process that can be monitored noninvasively using ^{31}P MRS (2, 3). Studies in cell-free extracts have shown that racemic (±)-gossypol can inhibit a variety of mitochondrial and cytoplasmic enzymes, including hexokinase and lactate dehydrogenase (4, 5), GST and lipooxygenase (5–7), Ca^{2+}-dependent protein kinase C (8, 9), and nuclear enzymes such as DNA polymerase (α and β) and topoisomerase II (10, 11). It is not

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ABBREVIATIONS: MRS, magnetic resonance spectroscopy; GST, glutathione-*S*-transferase; CaM, calmodulin; PKC, protein kinase C; EM, electron microscopy; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PS, phosphatidylserine.

yet clear whether the mitochondrial uncoupling and ATPase properties of gossypol are related to its ability to inhibit key intracellular enzymes or the extent to which either of these properties contributes to its antitumor effects.

We have tried to relate the antitumor and enzyme-inhibiting properties of gossypol by comparing the effects of its purified optical (+)- and (−)-isomers. Initial studies suggested that (−)-gossypol produces at least 2-fold greater antifertility and antiproliferative effects *in vitro* and *in vivo*, compared with the effects of (+)-gossypol (12–14). In cell-free or serum-free assay conditions, gossypol inhibitory effects on cell mitochondria and some enzyme systems may be nearly equal for the two enantiomers (15). We have investigated this discrepancy by correlating the antitumor and antimitochondrial properties of (+)- versus (−)-gossypol with their effects in human carcinoma cell lines on selected RNA transcripts (from the heat shock protein-70 gene, c-*myc* oncogene, and β2-microglobulin gene) as well as on several isolated enzyme systems (PKC, Ca^{2+}-dependent CaM activity, and GST isozymes) that may be involved in tumor growth regulation and resistance to therapy.

Materials and Methods

Growth inhibition of human tumor cell lines and bone marrow stem cells. Gossypol enantiomers [(+)- and (−)-] were purified as previously described (16). The antiproliferative effects of each enantiomer were determined for each of the previously described cell lines, which were originally derived from breast (MCF-7, T47-D), ovarian (OVCAR-3), colon (HCT-8), and pancreatic (MiaPaCa) neoplasms (2). These antitumor effects were also compared with enantiomer effects on the clonogenic growth of normal human bone marrow stem cells.

For the assessment of tumor cell line growth inhibition, cells (5×10^4) were plated on day 0 into replicate 25-cm^2 sterile plastic flasks (Costar, Cambridge, MA) and allowed to attach for 24 hr before drug addition on day 1. The drugs were added from ethanol-dimethyl sulfoxide-containing stock solutions, to a final vehicle concentration ≤0.1% ethanol and ≤0.002% dimethyl sulfoxide. Drug exposures were continuous until cells were harvested on day 6–8, when control culture growth was still in logarithmic phase and when the total cell count had reached $1–2 \times 10^6$ cells/flask. Trypsin-harvested cells were counted on a model ZB1 counter (Coulter Electronics, Inc., Hialeah, FL), and the results of these growth inhibition studies were reported as either the mean ± standard deviation of cell number or the mean percentage of control cell counts. All experiments were repeated at least once for verification.

To culture bone marrow stem cells, the freshly aspirated normal bone marrow from three consenting patients was injected into sterile tubes containing preservative-free heparin (1000 IU). The marrow suspension (7 ml) was layered onto 3 ml of lymphocyte separation medium (Bionetics Laboratory Products, Kensington, MD) and centrifuged, and the stem cell layer was removed. Remaining erythrocytes were removed by hypotonic shock, and the nucleated cells were then washed twice with McCoy's medium 5A (GIBCO Laboratories) containing 10% fetal calf serum. Bone marrow stem cell colonies were grown from the nucleated cells (10^5), which were plated into replicate 35-mm wells using the standard bilayer-agar method previously described (2). Colony formation was microscopically quantitated 9–12 days later, and the cloning efficiency of vehicle-treated marrow cells processed in this manner was about 0.1%.

EM and ^{31}P MRS. EM was performed on carcinoma cells after treatment in culture. Following vehicle or drug treatment, monolayer cells were harvested, rinsed free of medium, fixed in cold 2.5% glutaraldehyde and 0.1 M sodium cacodylate, and then treated with 1.5% osmium tetroxide. The fixed cells were later suspended in 0.5% uranyl acetate in veronal acetate buffer, this was followed by dehydration in graded ethanol and embedding in Polybed 812 (Pelco Inc., Redding, CA). Thin sections were poststained with uranyl acetate and lead citrate, examined at 80 KV in a JEOL 100S electron microscope, and then photographed.

^{31}P MRS was performed on cultured carcinoma cells using a home-built 5.6 T spectrometer configured about a Nicolet 1180 computer, as previously described (2). Spectra were obtained using a 60° tip angle, 25-μsec pulse width, spectral width of 10 KHz, recycle time of 2.2 sec, and quadrature detection over 4000 data points in each channel; data were processed using 20–30 Hz of line broadening. Methylenediphosphonic acid (0.5 M) in Tris buffer, pH 7.4, was used as an external reference. Treated cells (5×10^8) were pelleted loosely into a 10-mm diameter glass tube and spectra were recorded at 4° within 20 min of culture harvest as described (2). Spectral peak areas were calculated by fitting computer-generated peaks to the acquired spectrum using Nicolet software. The ratios of ATP/P_i peak areas were calculated to compare drug-treated with vehicle-treated control values.

RNA transcript levels. Total cellular RNA was extracted from logarithmic phase cultures of treated and control carcinoma cells (5×10^6) by guanidine isothiocyanate-cesium chloride gradient centrifugation, electrophoresed (10 μg/lane) for Northern blot hybridization into 1% agarose-formaldehyde gels, and transferred onto nitrocellulose filters as previously described (17). Probes containing c-*myc* or β_2-microglobulin sequences (16) or heat-shock protein cDNA sequences for *hsp*-70, *hsp*-90, and *hsp*-27 (18) were ^{32}P-labeled by random primer extension and individually hybridized (10^7 cpm) to the bound RNA. After autoradiography, filters were stripped and rehybridized to compare c-*myc*, β_2-microglobulin, and *hsp* transcripts from the same blotted RNA samples.

GST Isozymes. To purify GST-α and GST-μ, normal human liver was pulverized at −70° and homogenized in 50 mM Tris·HCl, pH 7.5, 0.5 M NaCl, followed by centrifugation at 27,000 × *g*. The supernatant was passed through a DEAE-cellulose column (Whatman DE-52) equilibrated with extraction buffer and was frozen in aliquots at −70° for later use. Aliquots of 25 ml (approximately 200 IU) were thawed, desalted on a 160-ml column of Sephadex G-25 (Pharmacia), and applied to an 85-ml column containing a mixture of 25 ml of *S*-hexyl-GSH-agarose and 60 ml of GSH-agarose (Sigma). The column was washed with 200 ml of 0.2 M NaCl in 50 mM Tris·HCl, pH 7.5, and the GST was eluted with 50 mM Tris·HCl, pH 9.6, containing 10 mM GSH and 0.2M NaCl. Fractions containing GST activity were pooled and dialyzed against 10 mM KPO_4, pH 6.5, and the near-neutral and basic GST isozymes were purified on a 45-ml hydroxylapatite column. The first peak, containing GST-μ, and the second peak, containing GST-α, were pooled separately, dialyzed against 10 mM KPO_4, pH 7.5, and frozen at −50° aliquots for later use. GST-π was purified from human placenta using the same protocol until reaching the GSH-affinity column step, after which the enzyme was dialyzed against 10 mM Tris·HCl, pH 7.5, loaded onto a 30-ml DEAE-cellulose column equilibrated with the same buffer, washed with 100 ml of equilibration buffer, and eluted with a 0–400 mM NaCl gradient in starting buffer (150 ml total). Fractions containing GST activity were pooled, dialyzed against 10 mM KPO_4, pH 7.5, and frozen. All GST isozymes were shown to be homogeneous and free of contamination by protein electrophoresis and Western blotting, using antibodies raised against each of the three classes of human GST. The absolute activities for the purified GST isozymes were GST-α = 11 nmol/min/mg, GST-μ = 11 nmol/min/mg, and GST-π = 13 nmol/min/mg. The effect of gossypol enantiomers on the purified isozyme activities was assayed under the following conditions: 1 mM GSH, 1 mM 1-chloro-2-dinitrobenzene (Sigma), in 0.2 M potassium phosphate buffer, pH 6.5. Gossypol (or 10 μl of vehicle) was added before enzyme addition, and the assays were performed at 25° using a Gilford Response spectrophotometer to detect product formation.

PKC and CaM-stimulated activity. PKC was purified from drug-resistant human cancer cell lines as previously detailed (19). The activity of PKC in the presence or absence (vehicle only) of gossypol enantiomers was determined by measuring incorporation from [γ-^{32}P]

ATP (3000 Ci/mmol) into lysine-rich histone under triplicate reaction conditions controlled for linearity with respect to incubation time and enzyme. The reaction mixture consisted of 40 μg of histone (type III-S; Sigma), 2.5 μg/ml diolein with or without 1.25 μg/ml PS (Sigma), and 50 μl (about 1 μg) of PKC enzyme, in 20 mM Tris·HCl containing 10 mM $MgCl_2$ and 0.5 mM $CaCl_2$, pH 7.5. The reaction was initiated by the addition of 20 μM ATP containing 4.4×10^6 cpm of [^{32}P]ATP in a final volume of 200 μl. After a 10-min incubation at 30°, reaction aliquots (25 μl) were spotted onto Whatman P81 cation exchange paper, washed three times in 75 mM phosphoric acid, and counted in a Beckman LS 7500 liquid scintillation counter. Basal PKC activity in the absence of PS was 0.10 (±10%) pmol of P_i/mg of protein/min, and PS stimulated PKC enzymatic activity 2- to 3-fold. Drug had no effect on basal enzymatic activity, and in the presence of PS the drug effect was recorded as the mean percentage of vehicle-treated and PS-stimulated PKC activity (100% control).

The activity of purified bovine brain CaM (CalBiochem) in the presence or absence (vehicle only) of various concentrations of gossypol enantiomers was determined by the ability of 10 units of CaM to stimulate a Ca^{2+}-CaM-dependent form of cyclic nucleotide phosphodiesterase. The phosphodiesterase was prepared, as previously detailed, by disruption of rat cerebral tissue in a sucrose/Tris/glycine/EGTA buffer (pH 7.6), followed by high speed centrifugation, dialysis, and electrophoresis of the enzyme-enriched supernatant (20). The cyclic AMP phosphodiesterase activity, as assayed by the luciferin-luciferase method, was 70 nmol of cyclic AMP hydrolyzed/mg of protein/min. CaM consistently produced 4- to 8-fold activation of this preparation of phosphodiesterase under control treatment conditions and the results from the drug treatment conditions were recorded as the mean percentage of control (100%) treatment value (20).

Results

Fig. 1 shows that a continuous exposure to (+)- or (−)-gossypol inhibited growth of human breast (T47-D), pancreatic (MiaPaCa), ovarian (OVCAR-3), and colon (HCT-8) cancer cell lines in a dose-dependent manner. Drug concentrations necessary to produce 50% growth inhibition (IC_{50}) of these cancer cells ranged between 1.5 and 4.0 μM for (−)-gossypol and 15 and 25 μM for (+)-gossypol. All but one of the cell lines were completely growth arrested by a 5 μM dose of (−)-gossypol; additional experiments showed that even 25 μM (+)-gossypol was insufficient to produce this same degree of growth arrest, which could, however, be achieved by a 50 μM dose of (+)-gossypol. The clonogenic stem cells from the three normal human bone marrow preparations appeared to be 5-fold more resistant than the carcinoma cell lines to (−)-gossypol, with an IC_{50} value ranging between 10 and 20 μM. Thus, both the marrow stem cells and the carcinoma cell lines required a 5- to 10-fold greater dose of (+)-gossypol to achieve the same degree of growth inhibition as for (−)-gossypol.

Comparison between the carcinoma cell lines suggested that differences in sensitivity to (−)-gossypol were more pronounced at concentrations exceeding the IC_{50} values. In Fig. 2, normal MCF-7 cells were compared with their Adriamycin-resistant subclone, MCF/Adr, which is known to possess the pleotropic characteristics of multidrug resistance, including overexpression of the *mdr*-1 gene (21). Previous studies had suggested that random selection of MCF-7 subclones did not, in itself, result in cells with altered drug or hormone sensitivities (22). Like the other carcinoma cell lines, MCF-7 and MCF/Adr cells were not significantly inhibited by (+)-gossypol doses up to 10 μM. However, with administration of 5 μM (−)-gossypol, MCF-7 cells were inhibited to 4% of control growth, whereas MCF/Adr cells were only marginally inhibited, to 81% of control growth. This resistant pattern of MCF/Adr response persisted for (−)-gossypol doses up to 10 μM, suggesting that the increased activity of GST and PKC associated with this Adriamycin-resistant subclone could also be associated with increased gossypol resistance (21).

With the continuous administration of 10 μM (−)-gossypol, most cell lines were permanently and completely growth arrested; a limited 24-hr exposure to this same dose also prevented further cell proliferation for at least 4 days. There was no

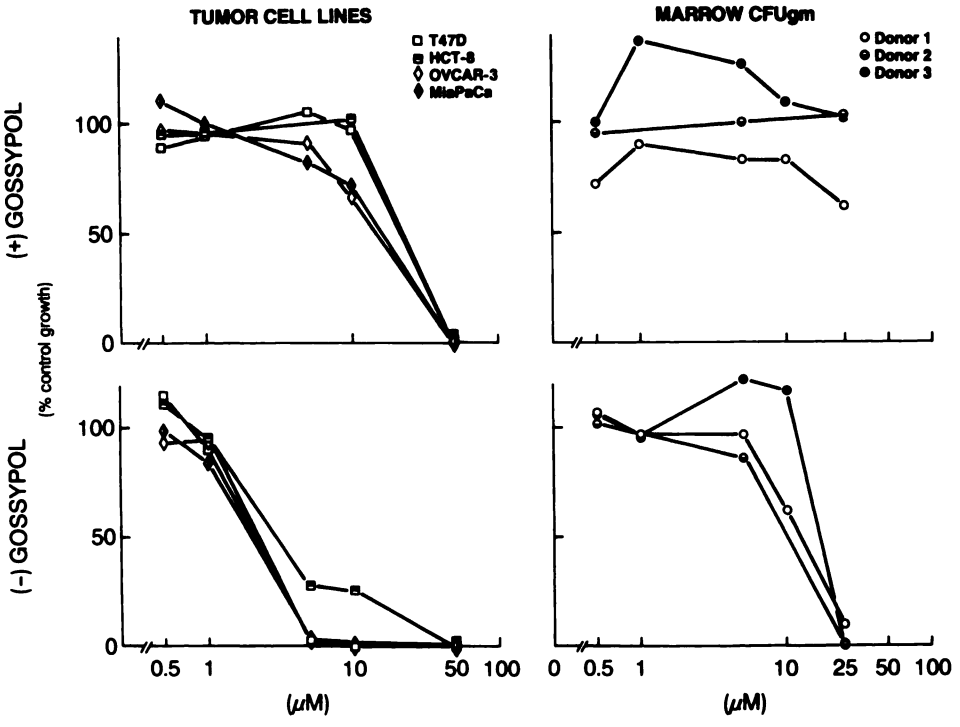

Fig. 1. Dose-dependent *in vitro* growth inhibition of human tumor cell lines and normal marrow stem cells (colony-forming units-granulocyte/macrophage) (*CFUgm*) by gossypol enantiomers. Tumor cells (5×10^4) are plated on day 0 into triplicate flasks and the drug is added on day 1 for a continuous exposure over 6–8 days, after which total cells are counted and recorded as the mean percentage of control (vehicle-treated) cell counts. For colony-forming units-granulocyte/macrophage growth, nucleated marrow cells (1×10^5) are cloned in replicate wells by standard bilayer-agar technique, in the presence of drugs or vehicle, and colonies of 15 or more cells are counted and recorded as the mean percentage of control colony growth. Control cloning efficiency is about 0.1%.

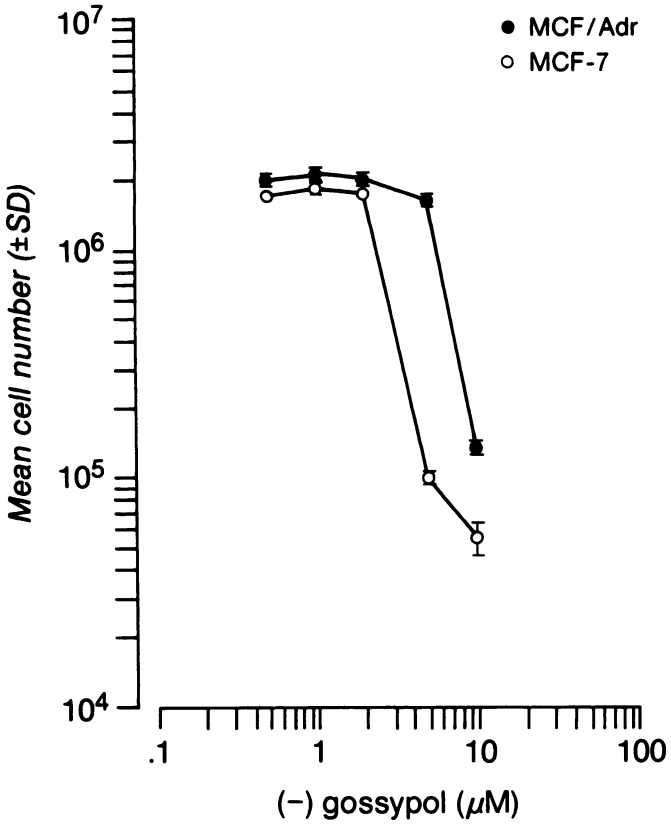

Fig. 2. (−)-Gossypol effects on growth of MCF-7 breast cancer cells and the multidrug-resistant subline MCF/Adr. Monolayer MCF-7 (○) and MCF/Adr (●) cells are grown, drug treated, and counted as described in Fig. 1. Counts from replicate flasks and two different experiments are averaged and recorded as the mean ± standard deviation of total cell counts for each treatment condition.

visible morphological change in the (−)-gossypol-treated cells immediately after 24 hr of exposure, when the monolayer cultures were examined under phase-contrast microscopy; however, ultrastructural analysis at this time revealed that extensive mitochondrial damage had occurred in the (−)-gossypol-treated cells. Fig. 3 shows control (vehicle-treated) and (+)-gossypol (10 μM for 24 hr)-treated MiaPaCa cells with normal nuclear and cytoplasmic appearance and intact mitochondria. After 24 hr of (−)-gossypol treatment, however, the mitochondria completely lost their cristae and could barely be recognized due to their distorted membrane structure and extensive vacuolization. Despite this severe mitochondrial damage, the cytoplasmic and nuclear EM appearance of these cells was otherwise normal, consistent with their normal phase-contrast microscopic appearance after 24 hr of treatment with (−)-gossypol.

In view of the ultrastructural evidence of mitochondrial destruction, ^{31}P MRS was performed to detect changes occurring in intracellular high energy phosphate levels after treatment with either (+)- or (−)-gossypol. Fig. 4 illustrates the spectral changes observed in T47-D cells treated with gossypol for 24 hr. (−)-Gossypol (10 μM) caused a profound reduction in nucleotide triphosphate (predominantly ATP) levels, associated with an increase in P_i, similar to our earlier ^{31}P MRS results using a commercial preparation of racemic gossypol (2, 3). The same dose of (+)-gossypol had little effect on ATP but slightly increased the P_i spectral peak in these cells. Table 1 compares 24-hr treatment effects on the ATP/P_i peak areas in four of the carcinoma cell lines treated with identical doses of the enantiomers. Although 10 μM (+)-gossypol was minimally cytotoxic for two of the five carcinoma cell lines studied (see Fig. 1), 24-hr exposure to (+)-gossypol reduced ATP/P_i levels only in the T47-D cells. In MiaPaCa cells, the same dose of (+)-gossypol reproducibly enhanced ATP/P_i content. In contrast, (−)-gossypol reduced ATP/P_i levels in all the cell lines, and the magnitude of this reduction varied from 69% to <5% of vehicle-treated control levels. Of note, the contrast between enantiomer effects on ATP/P_i pools in MiaPaCa cells was consistent with their mitochondrial effects noted on EM. In Table 1, the measured ATP/P_i changes in each cell line were also expressed as ratios of the potency of (−)-gossypol relative to (+)-gossypol, and these differences ranged from 1.6 to >50-fold. The variation in (−)-gossypol potency had a close rank order correlation with the sensitivity of these cell lines to (−)-gossypol (1–10 μM); that is, the more sensitive cells (MiaPaCa, T47-D) showed the greatest reduction in high energy phosphate levels, compared with the less sensitive cells (OVCAR-3, HCT-8).

Mitochondrial uncoupling, or other nonmitochondrial cytotoxic effects induced by gossypol, could lead to the altered expression of critical RNA transcripts. RNA extracted from cells exposed for various intervals (0–60 hr) to (+)- or (−)-gossypol (5 μM) was analyzed by Northern blot hybridization to detect changes in the expression of heat-shock genes (*hsp*-70, -90, and -27), a growth-related oncogene (c-*myc*), and a constitutively expressed structural gene (B_2-microglobulin). In Fig. 5, it can be seen that MCF-7 levels of B_2-microglobulin mRNA exhibited little change under all gossypol treatment conditions. When lane-loading differences are taken into account, this figure suggests that both (+)- and (−)-gossypol isomers produced equivalent biphasic changes in c-*myc* expression, with an initial decline in mRNA levels at 12 hr, followed by a return above baseline levels after 24 hr. These isomer-independent effects on c-*myc* expression were not studied further but served as an additional set of controls to illustrate the isomer-specific effects of gossypol on *hsp*-70 transcript levels. As shown in Fig. 5, (−)-gossypol produced a transient 4- to 5-fold increase in *hsp*-70 mRNA, coincident with a heat (41° for 15 min)-stimulated 10-fold increase in *hsp*-70 mRNA 6 hr after culture treatment, relative to vehicle- or (+)-gossypol-treated cells. Repeat analyses showed that this 6-hr increase in *hsp*-70 mRNA ranged from 2- to 8-fold over baseline MCF-7 *hsp*-70 levels and followed a steep dose dependency, with induction beginning at concentration above 2.5 μM and peaking at 10 μM (−)-gossypol. Probing these same blots for expression of other heat shock gene products revealed that the magnitude of (−)-gossypol induction of *hsp*-90 and *hsp*-27 mRNA averaged ≤2-fold.

The enzyme-inhibiting properties of each enantiomer were compared in cell-free assays using pure or partially purified proteins. Fig. 6 compares (+)- versus (−)-gossypol inhibition of the cationic (α), neutral (μ), and anionic (π) isozymes of GST. As shown, the dose-dependent inhibition of GST-μ was about equal for the two enantiomers (μ $IC_{50} \approx 2.5$ μM); however, inhibition of the other two isozymes yielded approximately 3-fold lower IC_{50} values for (−)-gossypol (α $IC_{50} = 1.6$ μM; π $IC_{50} = 7.0$ μM). Similar studies compared gossypol antagonistic effects on CaM and PKC activities. Both isomers were found

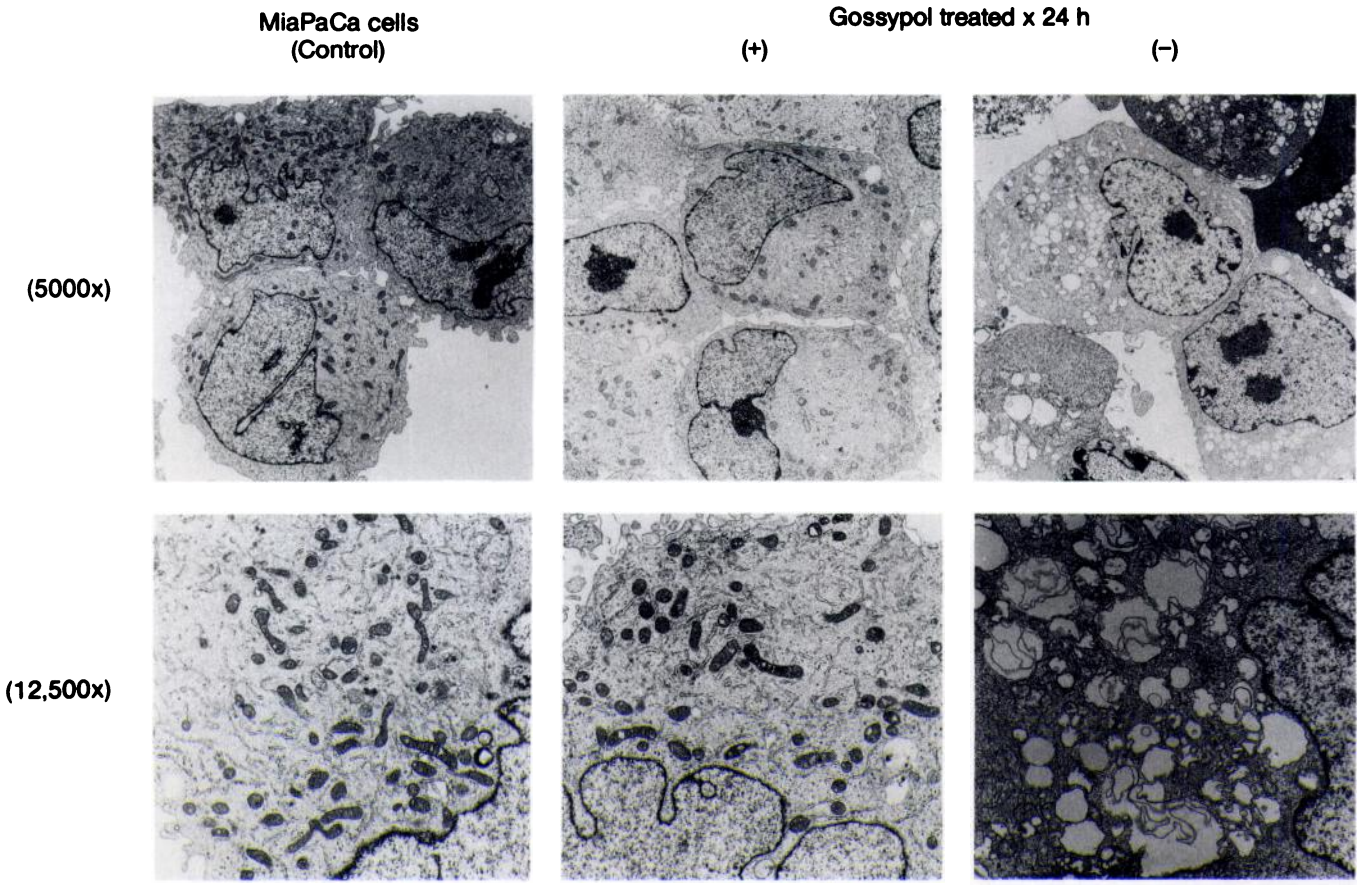

Fig. 3. EM appearance of treated MiaPaCa cells. Cultured cells are exposed for 24 hr to ethanol/dimethyl sulfoxide vehicle (control) or to a 10 μM dose of either (+)- or (−)-gossypol. Photographs taken after standard preparation of cells for EM show ultrastructural detail at low (5,000×) and high (12,500×) power magnification.

to have potent, dose-dependent, and near equivalent CaM-inhibitory properties, with IC_{50} = 6.0 and 4.0 μM for the (+)- and (−)-enantiomers, respectively; as well, both isomers showed potent, dose-dependent, and similar inhibitory effects on PKC activity, with IC_{50} = 1.5 and 2.4 μM for (+)- and (−)-gossypol, respectively.

Discussion

Our *in vitro* studies demonstrate that the nearly 10-fold more potent antiproliferative effects of (−)-gossypol relative to (+)-gossypol extend to a wide variety of human carcinoma cell lines as well as to normal human hematopoietic stem cells grown in culture. IC_{50} values of (−)-gossypol ranging from 1.5 to 4.0 μM for these cell lines are consistent with values reported for other cell lines derived from human reproductive tract cancers (14) and are about 2-fold lower than earlier determined IC_{50} values for the same cells tested with a commercial preparation of racemic gossypol (2).

EM observations suggested that the earliest ultrastructural change in cells sensitive to the antiproliferative effects of (−)-gossypol is the destruction of tumor mitochondria, occurring within 24 hr of drug exposure. This early and specific organelle damage likely results from gossypol-induced mitochondrial uncoupling, subsequent loss of the mitochondrial transmembrane proton gradient, activation of ATPase, and organelle swelling (2–4). With the observed resultant degree of structural damage to tumor mitochondria, it is not surprising that ^{31}P MRS detects pronounced (−)-gossypol-induced changes in high energy phosphate metabolism, manifested by a 1.6 to >50-fold differential reduction in the intracellular ratio of ATP/P_i relative to the effect of (+)-gossypol. Variability in the magnitude of the uncoupling and ATPase effect correlated with sensitivity of the cells to the antiproliferative effect of (−)-gossypol, implicating these early events in the antitumor mechanism of (−)-gossypol. Additional EM observations in cultured cells demonstrated mitochondrial swelling preceding membrane and cristae damage and appearing as early as 1 hr after drug administration; this was associated with early uncoupling and a measurable increase in the spectral P_i peak before any decline in the ATP peak was detectable (data not shown). A recent report measured early increases in the microviscosity of mitochondrial and microsomal membranes of human tumor cells sensitive to gossypol (23). As well, ultrastructural studies on late spermatids of rodents given contraceptive doses of gossypol have revealed similar pathognomonic mitochondrial changes that are specific for the (−)-isomer of this agent (24). Thus, the early and enantiomer-specific nature of our EM-observed mitochondrial damage appears to be a novel feature associated with both the antitumor and contraceptive properties of gossypol and is unlike the mode of cytotoxic injury induced by standard antiproliferative drugs, suggesting that gossypol belongs in a prototype class of antimitochondrial chemotherapeutic agents.

An interesting molecular correlate of the early antimitochondrial effects of (−)-gossypol, and occurring at doses exceeding

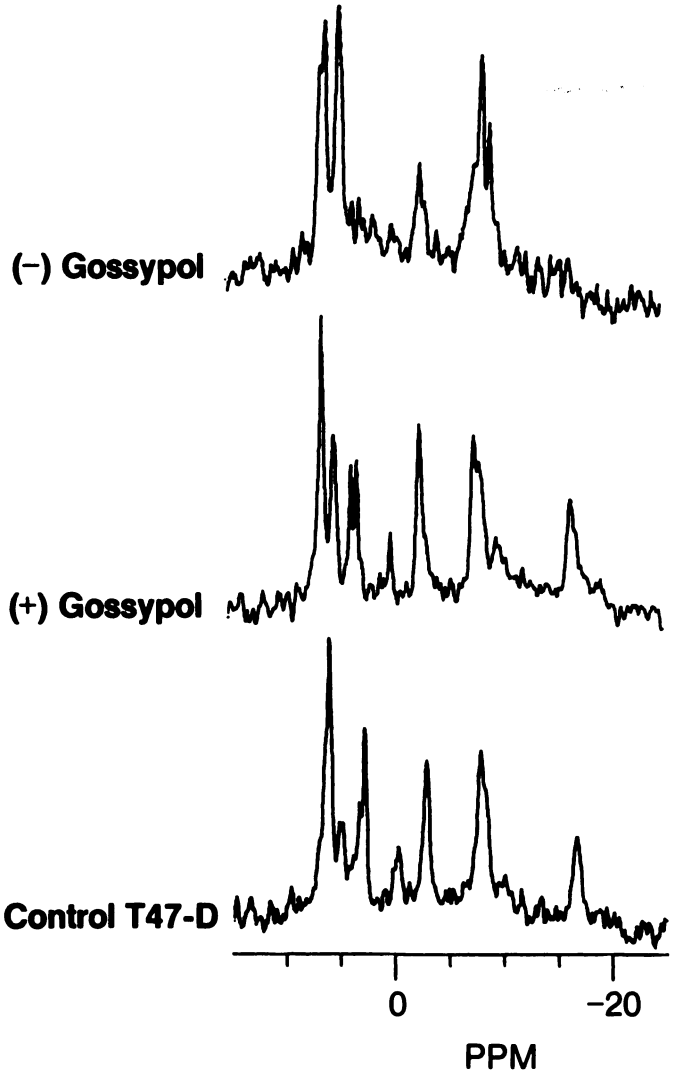

Fig. 4. ^{31}P MRS effects of gossypol treatment in cultured T47-D cells. Exponentially growing cells are exposed for 24 hr to ethanol/dimethyl sulfoxide vehicle (control) or to a 10 μM dose of either (+)- or (−)-gossypol. Spectroscopy is performed at 4° on freshly harvested cells, as described in Materials and Methods. The peak chemical shifts at +5.2 ppm (P_i) and −16.6 ppm (nucleotide triphosphates, predominantly ATP) show altered phosphorus spectra occurring as a result of treatment, relative to the control cell spectrum. In particular, the T47-D spectra indicate that (−)-gossypol produces an increase in P_i and a marked reduction in ATP, whereas (+)-gossypol produces only a slight increase in P_i. Measurement of the P_i and ATP peak areas enables the determination of ATP/P_i ratios.

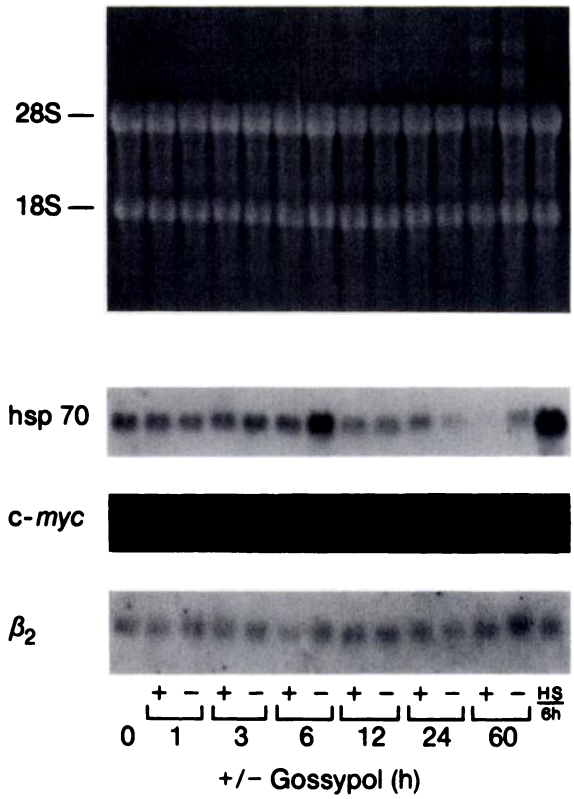

Fig. 5. Northern blot analysis of RNA transcript levels in tumor cells treated with (+)- or (−)-gossypol. MCF-7 cells are treated with 5 μM (+)- or (−)-gossypol for up to 60 hr and compared with vehicle-treated control cells (0 hr) or cells given a 15-min heat shock (*HS*) at 41° followed by a further 6-hr incubation at 37° before harvest. RNA samples (10 μg each) extracted from harvested cells are electrophoresed in agarose-formaldehyde gels, stained with ethidium bromide to compare ribosomal RNA bands (28 S and 18 S), membrane transferred, and hybridized with ^{32}P-labeled probes. The membrane is repetitively stripped, hybridized, and autoradiographed to detect the *hsp*-70, c-*myc*, and β_2-microglobulin transcripts bound to the same blot. Unequal lane loading accounts for the apparent variation in (+)- versus (−)- transcript levels at 60 hr.

TABLE 1

Treatment effects on intracellular ATP/P_i ratios

Carcinoma cell lines are treated for 24 hr with a 10 μM dose of (+)- or (−)-gossypol; ^{31}P MRS determination of the ATP and P_i peak areas is used to calculate and compare ATP/P_i ratios for treated and control cells.

Cell line	ATP/P_i		
	treated/control		(+)-Gossypol/(−)-gossypol
	(+)-Gossypol	(−)-Gossypol	
MiaPaCa	2.74	<0.05	>50
T47-D	0.36	<0.05	> 7
Ovcar-3	1.07	0.69	1.6
HCT-8	0.94	0.53	1.8

the IC_{50} antitumor values, is the increased expression of *hsp*-70 transcripts measured 6 hr after (−)-gossypol administration. Investigators studying thermogenesis in nonhibernating mammals have shown that temperature-induced physiological uncoupling of brown fat mitochondria increases cellular caloric output sufficient to heat local adipose tissue and its circulating blood up to 6° (25). This natural uncoupling mechanism is mediated by thermogenin, an anion-conducting pore protein located within the inner mitochondrial membrane (26). Similar non-thermogenin-containing OH^-- and P_i-conducting pores are also present in liver and heart mitochondria; they may account for rapid changes in mitochondrial volume, as observed in a variety of normal and pathological states, and they also appear to respond to a variety of chemiosmotic signals, including thyroid hormone, Ca^{2+}, pH, and oxygen tension (26). If (−)-gossypol were to uncouple tumor cell mitochondria by disrupting these anionic channels, then the intracellular heat production induced by this uncoupling would be sufficient to stimulate synthesis of the most sensitive of heat shock gene products, *hsp*-70. For carcinoma cells transiently exposed to a 4° increase in culture temperature, the expected increase in *hsp*-70 mRNA occurred within 6 hr and reached a level slightly exceeding that induced by exposure to 5 μM (−)-gossypol. Dinitrophenol, a

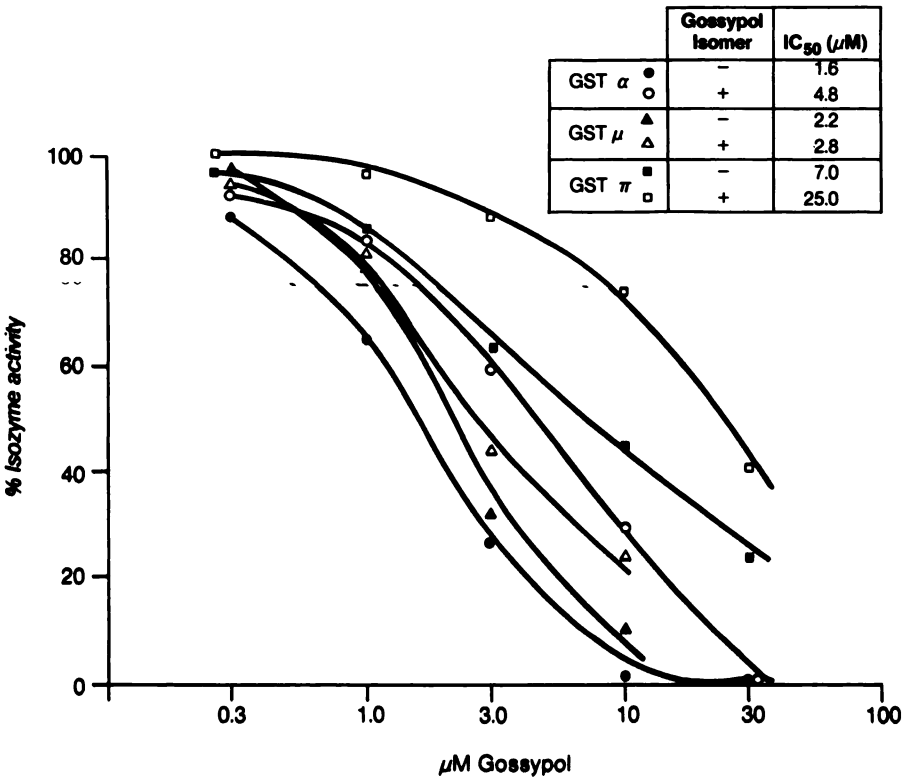

Fig. 6. Inhibition by gossypol isomers of GST isozymes (GST-α, -μ, and π). Freshly prepared and purified GST isozymes are assayed for enzymatic activity in the presence of varying isomer concentrations, as described in Materials and Methods. From the plots of percentage of isozyme activity, gossypol concentrations resulting in 50% activity (IC_{50}) are determined for each isozyme.

well studied uncoupling agent, is also known to induce thermotolerance and an increase in *hsp*-70 expression; as well, various other chemical and physical agents, not all of which directly modify mitochondrial function, can induce the synthesis of this class of heat shock gene product (27).

Previous reports have attributed the differential antifertility and antiproliferative effects of gossypol enantiomers to the higher serum protein-binding affinity of (+)-gossypol, presumably resulting in a lower extracellular concentration of free (+)-gossypol and reducing its intracellular uptake (15). However, this mechanism cannot reconcile disparate observations of gossypol isomer effects, such as the more potent ability of (+)-gossypol to induce DNA strand breaks in human leukocytes (16), our observed isomer-independent effects on c-*myc* expression and inhibition of CaM and PKC activities, or the greater inhibition by (−)-gossypol of certain GST isozymes (whose activities were assayed under serum-free and cell-free conditions). Unexplored alternative hypotheses that might explain such variable isomer effects include the selective membrane sequestration of (+)- or (−)-gossypol by chiral-specific phospholipids (28) or the chiral nature of structure-function relationships involving the hydroxyl, aldehyde, and isopropyl moieties on each of the naphthyl rings that appear to mediate the contraceptive and antiproliferative activities of gossypol (11, 24). Reports indicating equal inhibition of lactate dehydrogenase and DNA polymerase activities by (+)- and (−)-gossypol (11, 15) are consistent with our results showing that the isomers have nearly equivalent inhibitory effects on CaM and PKC activity. Both enantiomers are potent CaM antagonists, with IC_{50} values that are 2- to 6-fold lower than those determined for various phenothiazines (20). The ability of gossypol to inhibit CaM activity has not been reported previously. The IC_{50} values for (+)- and (−)-gossypol inhibition of PKC (1.5–2.4 μM) are 15-fold lower than that previously reported for racemic gossypol and 50-fold lower than that determined for another potent PKC inhibitor, trifluoperazine (8). Structure-function studies demonstrating an influence of gossypol's hydroxyl and aldehyde groups on its *in vitro* ability to inhibit both PKC and CaM would establish an important connection with its known enantiomer-nonspecific effects on DNA polymerase (11). In contrast to these isomer-independent gossypol effects on CaM and PKC activities, two isozymes of GST appear to be able to distinguish one enantiomer from another, supporting the possible relationship between chiral-specific protein interactions and the enhanced antitumor potency of (−)-gossypol. Unlike GST-μ, GST-α and GST-π demonstrated 3-fold greater enzyme inhibition by (−)-gossypol when assayed under identical conditions. Because GST-μ and GST-α were purified during the same procedure using a single tissue source, this difference in enantiomer recognition by the two isozymes is not likely an artifact of the purification procedure or the assay. The IC_{50} values for GST-μ are identical to a previously reported value for racemic gossypol acetic acid; however, (−)-gossypol appears to have a 15- to 25-fold greater inhibitory effect than the reported IC_{50} values for this racemic compound on both the GST-α and GST-π isozymes (6). With the isomer-specific antimitochondrial and contraceptive properties of gossypol reportedly dependent on its isopropyl sidegroups (24), it would be of considerable interest to establish the chiral importance (or lack of) of this side group across the internaphthyl bond in mediating the enhanced inhibition of GST-α and π by (−)-gossypol.

Our *in vitro* assays add some support to, but do not prove, an association between the enzyme-inhibiting properties of (−)-gossypol and either its antimitochondrial or antitumor effects. We do provide two new examples (GST-α and GST-π) of

preferential protein recognition and enhanced enzyme inhibition by (−)-gossypol, which must be considered in contrast to earlier reports explaining the enhanced potency of (−)-gossypol on the basis of more avid protein binding ty (+)-gossypol (15). The potent enzyme-inhibiting properties of (−)-gossypol suggest several possible mechanisms and structure-function relationships that might account for its enhanced antitumor and antimitochondrial properties that will remain questions for future study. Of additional interest is the significant difference between (−)-gossypol's antiproliferative effects on the MCF-7 and MCF/Adr cell lines, because these syngeneic human breast cancer cells differ only by the coupled set of metabolic parameters associated with acquisition of the multidrug-resistant (*mdr*) phenotype (21). Included in this set of *mdr*-associated changes in MCF/Adr cells are the increased cytoplasmic activities of GST-π and PKC (19, 21), making it possible that the increased (−)-gossypol resistance of MCF/Adr cells is due to their increased intracellular content of these enzymes. Inhibition of these enzymes might serve to reverse the *mdr*-associated phenotype (19, 21); thus, it may be interesting to investigate the effect of (−)-gossypol on restoration of the sensitivity of MCF/Adr cells to various chemotherapeutic agents such as anthracyclines, *Vinca* alkaloids, and cisplatin. The potent ability of (−)-gossypol to inhibit GST, PKC, and CaM, along with its induction effects on an important class of heat shock proteins (which might adversely promote drug resistance), warrants a thorough investigation into the molecular and antiproliferative effects of (−)-gossypol in combination with other antitumor agents, particularly with regard to *mdr*-expressing human cancers.

References

1. Tuszynski, G. P., and G. Cossu. Differential cytotoxic effect of gossypol on human melanoma, colon carcinoma, and other tissue culture cell lines. *Cancer Res.* **44**:768–777 (1984).
2. Benz, C., C. Hollander, M. Keniry, T. L. James, and M. Mitchell. Lactic dehydrogenase isozymes, ^{31}P-magnetic resonance spectroscopy, and *in vitro* antimitochondrial tumor toxicity with gossypol and rhodamine-123. *J. Clin. Invest.* **79**:517–523 (1987).
3. Benz, C., M. Keniry, and H. Goldberg. Selective toxicity of gossypol against epithelial tumors and its detection by magnetic resonance spectroscopy. *Contraception* **37**:221–228 (1988).
4. Ueno, H., M. K. Sahni, S. J. Segal, and S. S. Koide. Interaction of gossypol with sperm macromolecules and enzymes. *Contraception* **37**:333–341 (1988).
5. Lee, C.-Y. G., Y. S. Moon, J. H. Yuan, and A. F. Chen. Enzyme inactivation and inhibition by gossypol. *Mol. Cell. Biochem.* **47**:65–70 (1982).
6. Tahir, M. K., C. Guthenberg, and B. Mannervik. Inhibitors for distinction of three types of human glutathione transferase. *FEBS Lett.* **181**:249–252 (1985).
7. Hamasaki, Y., and H-H. Tai. Gossypol, a potent inhibitor of arachidonate 5- and 12-lipooxygenases. *Biochim. Biophys. Acta* **834**:37–41 (1985).
8. Kimura, K., K. Sakurada, and N. Katoh. Inhibition by gossypol of phospholipid-sensitive Ca^{+2} dependent protein kinase from pig testis. *Biochim. Biophys. Acta* **839**:276–280 (1985).
9. Nakadate, T., A. Y. Jeng, and P. M. Blumberg. Comparison of protein kinase C functional assays to clarify mechanisms of inhibitor action. *Biochem. Pharmacol.* **37**:1541–1545 (1988).
10. Adlakha, R. C., L. J. Rosenberg, and P. N. Rao. Inhibition of DNA polymerase-*alpha* and ribonucleotide reductase by gossypol. *Proc. Am. Assoc. Cancer Res.* **26**:249 (1985).
11. Adlakha, R. C., C. L. Ashorn, D. Chan, and L. A. Zwelling. Modulation of 4′-(9-acridinylamino)methanesulfon-*m*-anisidide-induced topoisomerase II-mediated DNA cleavage by gossypol. *Cancer Res.* **49**:2052–2058 (1989).
12. Joseph, A. E. A., S. A. Matlin, and P. Knox. Cytotoxicity of enantiomers of gossypol. *Br. J. Cancer* **54**:511–513 (1986).
13. Lindberg, M. C., R. H. Naqui, S. A. Matlin, R. H. Zhou, G. Bialy, and R. P. Blye. Comparative anti-fertility effects of gossypol enantiomers in male hamsters. *Int. J. Andro.*. **10**:619–623 (1987).
14. Band, V., A. P. Hoffer, H. Band, A. E. Rhinehardt, R. C. Knapp, S. A. Matlin, and D. J. Anderson. Antiproliferative effect of gossypol and its optical isomers on human reproductive cancer cell lines. *Gynecol. Oncol.* **32**:273–277 (1989).
15. Tanphaichitr, N., L. M. Fitzgerald, and S. A. Matlin. Differential effects of (+) and (−)-gossypol enantiomers on mitochondrial functional and proliferation of cultured TM4 cells. *J. Andro.*. **9**:270–277 (1988).
16. Matlin, S. A. A. Belenguer, R. G. Tyson, and A. N. Brookes. Resolution of gossypol: analytical and large-scale preparative HPLC on non-chiral phases. *J. High Res. Chromatogr. Chromatogr. Commun.* **10**:86–91 (1987).
17. Santos, G., G. Scott, W. Lee, E. Liu, and C. Benz. Estrogen-induced post-transcriptional modulation of c-*myc* proto-oncogene expression in human breast cancer cells. *J. Biol. Chem.* **263**:9565–9568 (1988).
18. Hickey, E., S. E. Brandon, S. Sadis, G. Smale, and L. A. Weber. Molecular cloning of sequences encoding the human heat-shock proteins and their expression during hyperthermia. *Gene* **43**:147–154 (1986).
19. Palayoor, S. T. J. M. Stin, and W. N. Hait. Inhibition of protein kinase C by antineoplastic agents: implications for drug resistance. *Biochem. Biophys. Res. Commun.* **148**:718–725 (1987).
20. Lee, G. L., and W. N. Hait. Inhibition of growth of C6 astrocytoma cells by inhibitors of calmodulin. *Life Sci.* **36**:347–354 (1985).
21. Moscow, J. A., and K. H. Cowan. Multidrug resistance. *J. Natl. Cancer Inst.* **80**:14–20 (1988).
22. Liu, E., G. Santos, W. Lee, C. K. Osborne, and C. Benz. Effects of c-*myc* overexpression on growth and estrogen sensitivity of MCF-7 human breast cancer cells. *Oncogene* **4**:979–984 (1989).
23. Wu, Y.-W., C. L. Chik, and R. A. Knazel, An *in vitro* and *in vivo* study of antitumor effects of gossypol on human SW-13 adrenocortical carcinoma. *Cancer Res.* **49**:3754–3758 (1989).
24. Hoffer, A. P., A. Agarwal, P. Meltzer, R. Naqvi, and S. A. Matlin. Antifertility, spermicidal and ultrastructural effects of gossypol and derivatives administered orally and by intratesticular injections. *Contraception* **37**:301–331 (1988).
25. Smith, R. E., and B. A. Horwitz. Brown fat and thermogenesis. *Physiol. Rev.* **49**:330–408 (1969).
26. Selwyn, M. J. Holes in mitochondrial inner membranes. *Nature (Lond.)* **330**:424–425 (1987).
27. Li, G. C. Heat shock proteins: role in thermotolerance, drug resistance, and relationship to DNA topoisomerases. *Natl. Cancer Inst. Monogr.* **4**:99–103 (1987).
28. Tsai, M. D., R. T. Jiang, and K. Bruzik. Phospholipids chiral at phosphorus. 4. Could membranes be chiral at phosphorus? *J. Am. Chem. Soc.* **105**:2478–2480 (1983).

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