Different Pathways of Cell Killing by Gossypol Enantiomers

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Gossypol, a polyphenolic, aldehyde-containing constituent of cottonseed, produced partial responses (>50% reduction in tumor size) in some patients with advanced cancer and suppressed sperm as an antifertility agent for men. This action *in vivo* and its novel side effect profile suggest a specific mechanism of the action of gossypol. Using the random homozygous knockout approach of Li and Cohen (1), we developed a cell line resistant to killing by gossypol, but sensitive to methotrexate and doxorubicin. It showed stereospecific resistance to killing by (–) gossypol (ED_{50} 4.9 μ*M*) compared with wild type (ED_{50} 2.0 μ*M*). The resistant and wild-type cells were equally sensitive to (+) gossypol (ED_{50} 8.8 and 8.4 μ*M*, respectively), methotrexate, and doxyrubicin. We conclude that gossypol affects cells by a stereospecific pathway for (–) gossypol, possibly related to its selective effects, and a nonstereospecific pathway for (+) gossypol and higher concentrations of (–) gossypol. Further knowledge about the stereospecific pathway may lead to new therapeutic drugs. [Exp Biol Med Vol. 227(6):398–401, 2002]

Key words: gossypol; cancer; stereoselectivity

Racemic gossypol is a polyphenolic constituent of cotton seed that causes male infertility. It was originally studied in China as a possible oral contraceptive agent for men (2, 3), and was subsequently studied elsewhere (4). Hypokalemia was the major adverse event observed in China, but untreated healthy men in China have lower serum potassium concentration values than men elsewhere (5) due to the environment in China, not due to Chinese genes (6). In addition, reversibility did not always occur after the gossypol was stopped.

Other laboratory studies found racemic gossypol to be active against many cancer cell lines both *in vitro* and *in vivo* (7). Clinical trials of racemic gossypol given orally to patients with advanced cancer produced 10% to 15% partial response rates when it was given in a single drug salvage protocol (8–10). The major side effects were nausea and vomiting in the third month of treatment, or rashes earlier in the course of treatment. These toxicities are different from the usual toxicities of the present cytotoxic antineoplastic drugs. These different toxicities suggest that the pharmacologic action of racemic gossypol differs from that of the usual antineoplastic cytotoxic drugs. Gossypol may be the lead compound for a new class of antineoplastic drugs or a new class of antifertility drugs active in men. Understanding its mechanism of action at the molecular level may lead to a new site or pathway to interdict to develop new therapeutic agents for treating cancer or for control of male fertility.

Tissue culture studies in cancer and other cell lines and tissues have found the (–) enantiomer of gossypol has more potent cytotoxic effects than the (+) enantiomer (11–16). The (–) enantiomer is also the active antifertility enantiomer in *in vivo* tests in male hamsters (17–19). *In vitro*, both enantiomers are equally potent inhibitors of several enzymes (20, 21), stimulators of superoxide free radicals by microsomes (22), and are spermicidal *in vitro* (23). Thus, racemic gossypol may have two mechanisms of action, a selective action at low doses of the (–) gossypol and a nonselective action from higher doses of either enantiomer.

We applied a molecular genetic technique to develop and select a line of gossypol-resistant cells. This cell line displays resistance to the stereospecific killing by (–) gossypol while remaining fully sensitive to the nonspecific effects of (+) gossypol, thereby distinguishing between these two pathways.

Materials and Methods

Briefly, the random controlled homozygous knockout technique of Li and Cohen (1) was used to produce a cell line resistant to gossypol cell killing.

Methodology. *Infection of host cells by the gene search vector, pLLGSV.* pLLGSV is a pHHAM-derived retroviral vector lacking the 3′LTR promoter and enhancer and containing the (-geo-reporter gene. The SV40 T antigen

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minimal early promoter and 14 copies of the *Escherichia coli* lac operator are inserted 5′ to an adenovirus-derived splice acceptor sequence. The expression of (-geo in cells containing the provirus is dependent on transcription from the adjacent chromosomal DNA. Such expression allows the cells to be selected by G418 and stained by X-gal for the production of B-galactosidase.

Activation of the antisense promoter by the transactivation vector, pLLTX. The transactivator vector pLLTX was derived from pHCMVLAP348. LAP348 contains the operator-binding domain of the *E. coli* LacI repressor protein and the herpes simplex virus transactivation domain, VP16. LAP348 will activate *in trans* the antisense promoter in the integrated provirus. The production of antisense RNA will inactivate (perhaps incompletely) the adjacent gene. pLLTX contains a HyTK gene expression cassette, a fusion of a hygromycin resistance gene, and the herpes simplex virus thymidine kinase gene. Transfectants expressing HyTK resist treatment with hygromycin, but not ganciclovir. In the absence of HyTK expression, cells are hygromycin sensitive and ganciclovir resistant.

Gossypol selection. The batches of infected, transfected cells were pooled and cultured in 8 μ*M* gossypol with 2 m*M* glutathione to stabilize the gossypol. Fresh gossypol with glutathione was added to each change of the medium. Gossypol-resistant colonies were formed during 10 to 12 days of culture. Individual colonies were selected and individually cultured after about 3 weeks. (Wild-type [gossypol-sensitive] cells normally die after 2–5 days in these culture conditions.)

Reverse the resistance by eliminating the antisense promoter via Cre-mediated excision. pLLTX was engineered to allow ready excision of the transactivator by the Cre protein. The HyTK gene expression cassette is ligated upstream of the transactivator gene, and the segment was bracketed by loxP sites. Expression of Cre, a site-specific recombinase that acts on the loxP sites of pLLTX, removes the transactivator, which shuts off the antisense promotor and allows selection by ganciclovir.

The line of cells from one colony that reversed after transfection with CRE, T_5G_{31}, was used for all studies. The packaging cell line GP-E-86 was originally a gift from Dr. Bank (Columbia University, New York). pLLGSV and pLLTX were gifts from Dr. S. Cohen (Stanford University, Stanford, CA). pBS185 was purchased from Invitrogen (Carlsbad, CA).

Drug Concentration-Response Determination. Equal numbers of cells, about 2×10^4, were plated into each well of a 24-well culture plate with the surface modified for endothelial cell growth (Falcon, 353847). Various concentrations of the study drug were added (with glutathione at 2 m*M* when gossypol was the study drug), and the cells were cultured for 2 days except for cultures with methotrexate for which it was 1 day. Then, trypan blue staining was performed, and trypan blue excluding cells in the center field of the well were counted. An Olympus microscope was used with a 10-power objective lens and a 10-power ocular lens into which a 100-square grid was placed. The results are presented as the number of cells per 100 squares.

Racemic gossypol and the enantiomers were supplied through the World Health Organization and from Dr. S. Matlin (City University, London, UK). Additional racemic gossypol was purchased from Sigma (St. Louis, MO). Doxorubicin and methotrexate were obtained from The New York Hospital pharmacy, New York, NY. The ED_{50} values for (+) and (–) gossypol effects were determined using the program of Chou and Chou (dose-effect analysis with microcomputer: quantitation of ED_{50}, LD_{50}, synergism, antagonism, low-dose risk, receptor-ligand binding, and enzyme kinetics [manual and software]; Biosoft, Cambridge, UK, 1987).

Results

To establish a gossypol-resistant cell line, seven mouse cell lines were screened for gossypol sensitivity The most sensitive cell line, SVEC4-10EE2 (American Type Culture Collection, Manassas, VA; No. CRL-2167), derived from mouse endothelial cells, was chosen for this study. About 500×10^6 cells were exposed to virus and were then selected with G418. About 600,000 colonies were formed, indicating pLLGSV integrated in frame in transcriptionally active genes. These were selected with G418, transfected with pLLTX, and selected with hygromycin. Culture of these cells in 8 μ*M* of gossypol produced 14 gossypol-resistant cell lines. One of these, T_5G_{31}, was transfected with pBS185-Cre and the transfected cells were selected with ganciclovir. This cell line is T_5G_{31}-cre. The T_5G_{31} cells are smaller than wild type and grow slightly slower.

The gossypol concentration response relationships for survival after 2 days for the three cell types (wild type [wt], T_5G_{31}, and T_5G_{31}-cre) are shown in Figure 1. The T_5G_{31} cell line is resistant to gossypol, whereas the T_5G_{31}-cre cell line has recovered some of the original gossypol sensitivity.

The specificity of the resistance of T_5G_{31} cells to gos-

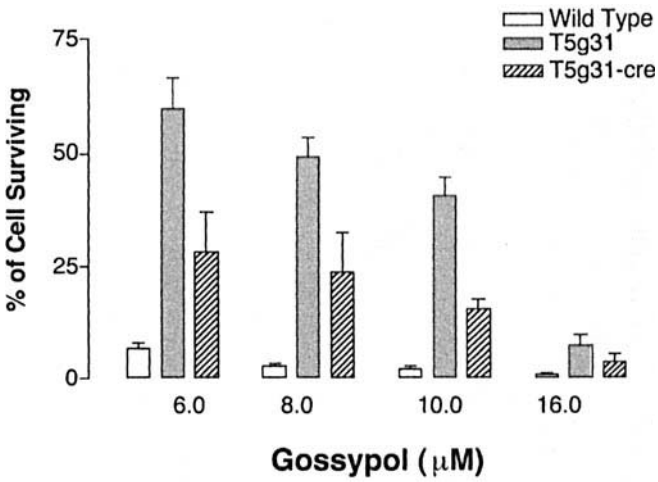

Figure 1. Survival of cells treated with gossypol (as a percentage of cells in 0 μ*M* gossypol) for 2 days. Data is mean ± SE of three experiments of three wells each at each concentration.

sypol was studied by assessing the concentration-response relationships of wild-type and T_5G_{31} cells to methotrexate (Fig. 2) and doxorubicin (Fig. 3). They show that T_5G_{31} cells are not resistant to these two cytotoxic drugs and may even be more sensitive to killing by these drugs. Thus, the resistance to gossypol is specific for gossypol.

The stereoselectivity of the resistance to gossypol was studied with a concentration-response experiment using (+) and (–) gossypol comparing wild-type and T_5G_{31} cells (Fig. 4). The increased gossypol resistance in T_5G_{31} is specific for the therapeutically more important (–) enantiomer. The dose-response curves are similar in the (+) enantiomer cultures. The ED_{50} value for (–) gossypol killing wild-type cells was 2.0 μM. For (–) gossypol and T_5 G_{31} cells, it was 4.9 μM. The ED_{50} for (+) gossypol and wild-type cells was 8.4 μM, and with T_5G_{31} cells, it was 8.8 μM.

Discussion

Using a molecular genetic technique (1), we have developed a cell line resistant to killing by (–) gossypol, but as sensitive as the wild-type parent mouse endothelial cell line to killing by (+) gossypol. This resistance is specific for (–) gossypol because these cells are not resistant to killing by methotrexate or doxorubicin. We think there is a genetically determined pathway by which (–) gossypol specifically kills cells. This pathway may relate to the effect of gossypol on sperm cell production or on its antineoplastic effect. It appears to be different from the pathway by which (+) gossypol kills cells because the T_5G_{31} cells are just as sensitive to (+) gossypol as the wild type. This indicates that there are two mechanisms by which racemic gossypol kills cells, a genetically controlled pathway specific for (–) gossypol and another pathway for (+) gossypol.

We found a 2.5-fold difference in ED_{50} values for (–) gossypol between sensitive wild-type cells and resistant T_5G_{31} cells. This is similar to the 2- to 3-fold difference in dose needed for antineoplastic or antifertility effect (20–30 mg/d) and the dose producing major toxicity (60–70 mg/d).

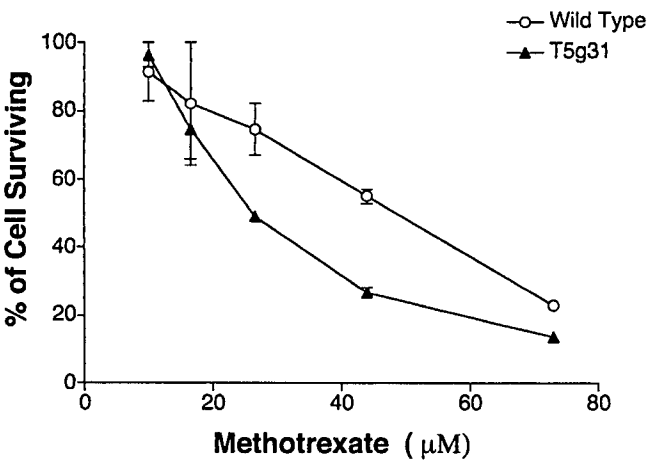

Figure 2. Survival of cells treated with methotrexate for 1 day (as percentage of cells in 0 μM methotrexate). Data is mean ± SE of three experiments of six wells at each concentration.

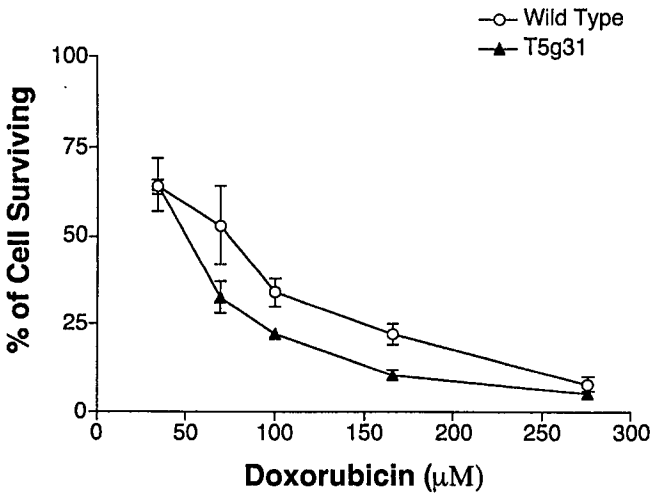

Figure 3. Survival of cells treated with doxorubicin for 2 days (as percentage of cells in culture without doxorubicin). Data is mean ± SE of three experiments of six wells at each concentration.

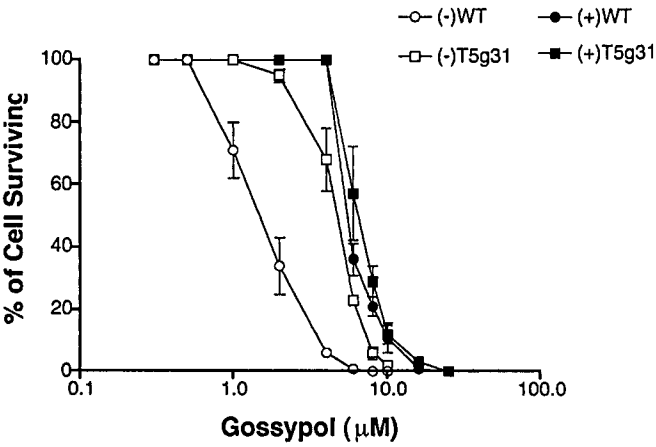

Figure 4. Survival of cells treated with gossypol enantiomers for 2 days. Data is mean ± SE of three experiments with nine wells at each concentration.

Another way to look at the data of Figure 4 is that at 4μM of (–) gossypol, 68% of the T_5G_{31} cells survived, whereas only 6% of wild-type cells survived—an 11-fold difference in survival rate.

The stereospecific pathway suppressed in the T_5G_{31} cells may relate to the specific effect of gossypol on sperm cell production, on the antineoplastic effect of gossypol, or both effects. With the clinical trial data showing that gossypol is an effective antifertility drug for men and that it has the same efficacy in a single drug salvage regimen for advanced cancer as many of our standard antineoplastic drugs, further study using genetic techniques to describe the mechanism of the stereospecific effects of gossypol is warranted. Knowledge of this mechanism may lead to a new class of antineoplastic or antifertility medications.

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