Action of Gossypol and Rhodamine 123 on Wild Type and Multidrug-resistant MCF-7 Human Breast Cancer Cells: $^{31}$P Nuclear Magnetic Resonance and Toxicity Studies

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ABSTRACT

The action of gossypol, a polyphenolic bisnaphthalene aldehyde, on a number of drug-sensitive and multidrug-resistant cell lines, in particular MCF-7 WT and MCF-7 ADR cells, was studied and compared to the effects of rhodamine 123. $^{31}$P nuclear magnetic resonance spectra of cells exposed to low concentrations of gossypol exhibited decreased levels of ATP, markedly increased levels of pyridine nucleotides, and decreased levels of glycerylphosphocholine. The latter effect may be related to the membrane viscosity-increasing effect of gossypol, whereas changes in the levels of pyridine nucleotides are probably due to an interference with NAD- and NADP-dependent enzymes. The effect of gossypol represents a rare example of selective and differentiated changes observed in $^{31}$P nuclear magnetic resonance spectra of cells following exposure to a drug: the effect was markedly different from that of rhodamine 123, which caused ATP depletion but no changes in the levels of glycerylphosphocholine or pyridine nucleotides. Also, the effects of gossypol and rhodamine 123 on glucose metabolism in the MCF-7 WT cells were different. Thus although both drugs caused a marked elevation of glucose uptake, an increase in lactate production exceeding that of glucose consumption, was obtained in the case of rhodamine 123. Significantly, multidrug-resistant cells exhibited cross-resistance to rhodamine 123. Multidrug-resistant cells showed increased levels of NADH in the case of rhodamine 123. Significantly, multidrug-resistant cells showed increased levels of NADH in the case of rhodamine 123, which emphasizes the attractiveness of the latter as a potential anticancer drug. The resistance to rhodamine 123 and sensitivity to gossypol was also observed with cells transfected with the MDR1 gene, showing that the difference in toxicity is mainly due to the different response to the P-170 drug efflux pump.

INTRODUCTION

Gossypol (Fig. 1), a polyphenolic aldehyde from the cottonseed, has attracted much attention as a potential male antifertility drug (1, 2). More recently, selective toxicity of gossypol against various cancer cell lines and experimental tumors has been demonstrated (3-9), suggesting its possible therapeutic use.

Considerable complexity of the biological actions of gossypol emerges from the literature. Because of its chemical nature as a highly reactive redox reagent and a Schiff base-forming agent as well as a polydentate metal-complexing ligand, gossypol can interact with cellular components in a variety of ways. Gossypol acts as a nonselective enzyme inhibitor in vitro (10) and can affect a number of cellular functions, including macromolecular synthesis (11, 12), ion transport (13), and properties of lipid membranes (4, 14, 15), glycolysis (16-18), respiration (19-22), and glucose uptake (23). Although it has recently been suggested that the toxic effect of gossypol on cancer cells is mechanistically similar to that of rhodamine 123 (Fig. 2) (6), which is an established antimitochondrial agent (24, 25), we found in the present study a considerable nonequivalence of these two compounds. Moreover, we found that multiple drug resistance, which is one of the major problems in cancer chemotherapy, is not capable of providing any significant protection against the toxic effects of gossypol. This finding adds to our interest in further evaluation of gossypol as a possible anticancer drug.

MATERIALS AND METHODS

Materials. IMEM supplemented with penicillin and streptomycin was obtained from the NIH Media Unit. Other media and auxiliaries were obtained from Gibco Laboratories (Grand Island, NY). Rhodamine 123 (laser grade) and MTT were obtained from Eastman Kodak Company (Rochester, NY) and Sigma Chemical Company (St. Louis, MO), respectively. Gossypol (acetic acid complex) was obtained from the Special Programme of Research, Development, and Research Training in Human Reproduction, WHO (Geneva, Switzerland).

Cells. Wild-type (WT) MCF-7 and the derived resistant cells (MCF-7 ADR) were established lines (26, 27) maintained at this laboratory; the cells were grown in IMEM containing 5% fetal calf serum. Monoclonal MCF-7 cells, MDR1-transfected MCF-7 cells (clone 10.2), and MDA-MB-468 human breast cancer cells (28) were obtained from the Lombardi Cancer Center, Georgetown University (Washington, DC), and grown in IMEM with 10% serum. Human cervical carcinoma (KB-1, KB-A1, and KB-V1) (29), human melanoma (FEMX and FEMX 4A P 550) (30) and Madin-Darby dog kidney tubular cells (MDCK and MDR-MDCK) (30) were obtained from the Laboratory of Molecular Biology, National Cancer Institute, and grown in IMEM containing 10% serum. BCAdr-19-transfected human breast cancer (MCF-7) cells (31) and human ovarian carcinoma (A2780 and A2780/CP70) (32) were obtained from the Medicine Branch, National Cancer Institute, and were grown as described previously (31, 32). Chinese hamster lung carcinoma (DC-3F and DC-3F/AD X) (33) and normal diploid myoepithelial breast cells (HS578Bst) (34) were obtained from Memorial Sloan-Kettering Cancer Center (New York, NY) and the American Type Culture Collection (Rockville, MD), respectively, and were also grown as described previously (33, 34). The cells were grown in 150 cm$^2$ flasks (Costar, Cambridge, MA) containing 30 ml of medium at 37°C in a humidified atmosphere containing 5% carbon dioxide and were harvested by trypsinization.

Procedures. Growth inhibition experiments were carried out in 96-well (flat bottom) microplates (Costar), and the amount of viable cells at the end of the incubation was determined using the MTT assay (35). Thus, 1000-1500 cells/well in 150 ml medium were plated and grown for 24 h, the chemicals were added (in 50 ml medium/well), and the cells were grown for an additional 5 days. After addition of MTT (50 ml, 2 mg/ml in phosphate-buffered saline), the plates were incubated for 4 h (6 h for the HS578Bst cells) and centrifuged at 800 × g for 10 min, the medium was removed, and the blue dye was dissolved in 200 ml of DMSO (1:1). The absorbance at 570 nm was measured with a Titertek Multiskan (Labsystems, Cambridge, MA).

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2. The abbreviations used are: IMEM, improved minimal Eagle’s medium; NMR, nuclear magnetic resonance; GPC, glycerylphosphocholine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; WT, wild type; ADR, Adriamycin-resistant.

3. R. Clarke et al., unpublished work.

4. M. M. Gottesman et al., unpublished work.
in 120 µl of dimethyl sulfoxide (20 min on an orbital shaker at 200 rpm). The absorbance was measured at 570 nm using a Vmax plate reader (Molecular Devices, Menlo Park, CA) interfaced to a Macintosh SE30 computer (Apple Computer, Inc., Cupertino, CA), also used for data analysis. Each point on growth curves was an average of at least six wells, and each assay was performed with at least two independent plates. The growth of the cells during the period of the growth inhibition experiments was logarithmic, as shown by independent determination of growth curves (in the absence of the drugs), also performed using the MTT assay.

Data on the dependence of growth inhibition on incubation time were obtained by plating 15 identical 96-well plates for each series of measurements and assaying 3 plates on each of days 2 to 6 (MTT method) for calculation of the 50% inhibitory concentration.

Glucose consumption and lactate production assays were performed with 80-85% confluent MCF-7 WT and ADR cells in ISO-cm² flasks containing 50 ml of fresh medium, medium containing 10 µM gossypol, or medium containing 10 µM rhodamine 123. Rhodamine was added from a stock solution dissolved in the medium; gossypol was added as an ethanol solution to a final concentration of ethanol in the medium of no more than 0.03%, and the same amount of ethanol was present in the control flasks. Samples (1 ml) were withdrawn from the flasks 5 times during a 25-h incubation period and analyzed for glucose and lactate.

Glucose and lactate determinations were carried out using the hexokinase and the lactate dehydrogenase assay kit, respectively (Sigma). Protein content in cell samples was determined after sonification (Heat Systems-Ultrasonics, Inc., Farmingdale, NY) using the bicinchonate assay kit (Pierce Chemical Co., Rockford, IL). Absorbance measurements were performed with a Shimadzu UV-160 spectrophotometer (Shimadzu Corp., Kyoto, Japan).

Nuclear Magnetic Resonance. The 1H NMR spectra were obtained at ambient temperature (20-22°C) with 10-mm nonspinning tubes on a Varian XL-400 spectrometer (Varian Associates, Inc., Palo Alto, CA), using 35-µs radiofrequency pulses (corresponding to 60-degree flip angle) with 3-s repetition delay. Usually, 4 K data points were acquired and Fourier transformed using line broadening of 20 Hz.

The cells for NMR experiments were grown to 80-85% confluency, the medium was replaced with fresh medium (30 ml) containing the appropriate chemical, and the cells were grown for additional 24 h; each experiment was paralleled with a control experiment with the same number of flasks. After harvest, the cells (usually 150-180 million corresponding to 5-6 flasks) were washed twice with cold (4°C) medium by centrifugation (7 min at 700 x g), and the pellet was resuspended in 2.0 ml of the medium and transferred to the NMR tube; in all cases the accumulation of data was started within 30 min after the harvest. Oxygen was bubbled gently through the suspension during data acquisition in order to oxygenate the cells and to prevent sedimentation.

All spectra were obtained at identical instrumental settings; three successive, independent blocks of 400 transients were collected and the spectra were transformed and inspected separately to assure that the samples did not change during the acquisition, after which the blocks were added and transformed together for final analysis and integration. The integrals were normalized to the protein content of the samples. At the end of acquisition (total of 1 h) the cells were checked for viability using the trypan blue exclusion test; the observed viabilities exceeded 95%.

NMR perfusion experiments with cells embedded in agarose gel threads were carried out as described previously at 37°C (36, 37).

RESULTS

Toxicity. Concentrations of gossypol and rhodamine 123 that cause inhibition of the growth of cells by 50% over a 5-day period were determined by MTT assay. The IC50 values (concentration that inhibits growth by 50%) are shown in the graphs.

Fig. 2. Effect of incubation time on 50% inhibitory concentration (IC50) of gossypol and rhodamine 123. Bars, SD.
incubation period, determined by the MTT assay (35), are shown in Table 1. Although the toxicity of gossypol may depend not only on the absolute drug concentration but also on the ratio between drug amount and the number of cells (19) and may vary according to the assay conditions, the range of 50% inhibitory concentration values obtained is closely similar to that found for other cancer cells in vitro, using different assays such as thymidine incorporation (3), protein production (8), and direct cell count (5, 6). As reported previously (6), rhodamine is somewhat more toxic than gossypol (Table 1). However, this applies only to drug-sensitive cells. The multidrug-resistant lines (MCF-7 ADR, BCAdr-19, MDR MCF-7, KB-A1, KB-V1, FEMX 4A P, DC-3F/AD X, MDR-MDCK) exhibited pronounced cross-resistance to rhodamine, whereas no or two very slight cross-resistance to gossypol was observed. The time profiles of toxicity of the two drugs were also different (Fig. 2), being possibly related to more rapid accumulation of rhodamine in the cells. Normal breast tissue (Hs578Bst) was less susceptible to gossypol toxicity than the breast cancer lines (Table 1).

NMR Spectra. Although cell perfusion techniques (36, 37) are in general most appropriate for monitoring of NMR spectra of cells influenced by xenobiotics, the apparently delayed effects shown in Table 1. Although the toxicity of gossypol may depend on the absolute drug concentration but also on the ratio between drug amount and the number of cells (19) and may vary according to the assay conditions, the range of 50% inhibitory concentration values obtained is closely similar to that found for other cancer cells in vitro, using different assays such as thymidine incorporation (3), protein production (8), and direct cell count (5, 6). As reported previously (6), rhodamine is somewhat more toxic than gossypol (Table 1). However, this applies only to drug-sensitive cells. The multidrug-resistant lines (MCF-7 ADR, BCAdr-19, MDR MCF-7, KB-A1, KB-V1, FEMX 4A P, DC-3F/AD X, MDR-MDCK) exhibited pronounced cross-resistance to rhodamine, whereas no or two very slight cross-resistance to gossypol was observed. The time profiles of toxicity of the two drugs were also different (Fig. 2), being possibly related to more rapid accumulation of rhodamine in the cells. Normal breast tissue (Hs578Bst) was less susceptible to gossypol toxicity than the breast cancer lines (Table 1).

Table 1 IC50 values for gossypol and rhodamine 123 (5-day incubation period)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Gossypol IC50 (μM)</th>
<th>Rhodamine IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7 WT*</td>
<td>3.4 ± 0.3</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>MCF-7 ADR4</td>
<td>4.3 ± 0.4</td>
<td>125 ± 4</td>
</tr>
<tr>
<td>BCAdr-19*</td>
<td>1.9 ± 0.2</td>
<td>46 ± 2</td>
</tr>
<tr>
<td>MCF-7 WT monoclonal*</td>
<td>1.7 ± 0.2</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>MDR MCF-7 clone 10.2*</td>
<td>1.7 ± 0.2</td>
<td>94 ± 2</td>
</tr>
<tr>
<td>MDA-MB-468*</td>
<td>4.8 ± 0.3</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>KB-3-1*</td>
<td>3.8 ± 0.4</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>KB-A1*</td>
<td>2.9 ± 0.1</td>
<td>&gt;235</td>
</tr>
<tr>
<td>KB-V1*</td>
<td>4.0 ± 0.3</td>
<td>&gt;235</td>
</tr>
<tr>
<td>FEMX*</td>
<td>2.4 ± 0.2</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>FEMX 4A P</td>
<td>5.0 ± 0.1</td>
<td>220 ± 11</td>
</tr>
<tr>
<td>A2780*</td>
<td>4.5 ± 0.2</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>A2780/CP70*</td>
<td>4.6 ± 0.2</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>DC-3F*</td>
<td>4.2 ± 0.1</td>
<td>132 ± 8</td>
</tr>
<tr>
<td>DC-3F/AD X</td>
<td>4.2 ± 0.1</td>
<td>&gt;230</td>
</tr>
<tr>
<td>MDRK*</td>
<td>6.0 ± 0.2</td>
<td>110 ± 3.3</td>
</tr>
<tr>
<td>MDR-MDCKM*</td>
<td>9.4 ± 0.5</td>
<td>&gt;235</td>
</tr>
<tr>
<td>Hs578Bst*</td>
<td>19.8 ± 2.3</td>
<td>1.9 ± 0.1</td>
</tr>
</tbody>
</table>

*IC50, 50% inhibitory concentration.
*Wild type cells.
*Mean ± SD.
*Resistant cells selected with Adriamycin (192-fold resistant) (27); MDR1 mRNA level more than 100 times higher than in the WT cells (31).
*Cells specifically transfected with the MDR1 gene, 14-fold resistant to Adriamycin, MDR1 mRNA level more than 153 times higher than in the WT cells (31).
*Cells specifically transfected with the MDR1 gene, 11-fold resistant to Adriamycin.
*Resistant cells selected with Adriamycin (43-fold resistant) (29).
*Expression of P-170 shown by various methods.
*Resistant cells selected with vinblastine (213-fold resistant) (29).
*Cells nonspecifically transfected with the MDR1 gene, 14-fold resistant to Adriamycin (32).
*Resistant cells selected with cisplatin (39-fold resistant, slight cross-resistance to Adriamycin) (32).
*Resistant cells selected with actinomycin D (2450-fold resistant) (33).
*Cells specifically transfected with the MDR1 gene (30).
*Normal diploid myoepithelial breast cells.

Typical 31P NMR spectra of MCF-7 WT, MCF-7 ADR, and MDA-MB-468 cells incubated for 24 h with 10 μM gossypol, together with the corresponding controls, are shown in Figs. 3–5. Assignment of the resonances follows from earlier studies (36–38). The treatment of MCF-7 WT cells with gossypol results in a dramatic increase of the level of pyridine nucleotides, together with a decrease of the level of the remaining metabolites (Fig. 3), most markedly phosphodiester (GPC and glycerylphosphoethanolamine). The spectra of MCF-7 ADR and MDA-MB-468 cells exhibit no visible phosphodiester peaks, but an increase of pyridine nucleotide peaks and decrease of the remaining signals is also observed (Figs. 4 and 5). The
average changes in the metabolite levels normalized to the protein content of the samples are shown in Table 2 (means of 2–6 measurements). Table 3 and Fig. 6 show similar data from treatment of the cells with rhodamine 123. Here no changes are observed with the MCF-7 ADR cells. In the MCF-7 WT cells a depletion of ATP is observed, but no depletion of GPC or increase of the level of pyridine nucleotides as with gossypol can be seen.

Glucose Metabolism. Since it is clear from earlier studies that both gossypol and rhodamine 123 can affect glucose metabolism, we investigated the effects of these drugs on glucose consumption and lactate production in MCF-7 WT and ADR cells. Thus, levels of glucose and lactate in the growth medium

Table 2 Levels of phosphorus-containing metabolites in breast cancer cells following incubation with 10 μM gossypol for 24 h (% of control)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>PME*</th>
<th>PDE</th>
<th>ATP</th>
<th>PN</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7 WT</td>
<td>92 ± 5</td>
<td>66 ± 10</td>
<td>78 ± 10</td>
<td>157 ± 6</td>
</tr>
<tr>
<td>MCF-7 ADR</td>
<td>74 ± 3</td>
<td>71 ± 10</td>
<td>116 ± 6</td>
<td></td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>93 ± 6</td>
<td>86 ± 6</td>
<td>122 ± 3</td>
<td></td>
</tr>
</tbody>
</table>

* PME, phosphomonoester; PDE, phosphodiester; PN, pyridine nucleotides. Mean ± SD.

Table 3 Levels of phosphorus-containing metabolites in MCF-7 cells following incubation with 10 μM rhodamine 123 for 24 h (% of control)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>PME*</th>
<th>PDE</th>
<th>ATP</th>
<th>PN</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7 WT</td>
<td>87 ± 14*</td>
<td>101 ± 7</td>
<td>74 ± 9</td>
<td>100 ± 7</td>
</tr>
<tr>
<td>MCF-7 ADR</td>
<td>104 ± 9</td>
<td>96 ± 9</td>
<td>96 ± 5</td>
<td></td>
</tr>
</tbody>
</table>

* PME, phosphomonoester; PDE, phosphodiester; PN, pyridine nucleotides. Mean ± SD.

Fig. 4. 31P NMR spectra of suspensions of MCF-7 ADR cells in the growth medium (2 ml). Bottom, cells incubated for 24 h with 10 μM gossypol (135 million cells); top, control (142 million cells). Peak assignments as in Fig. 3.

Fig. 5. 31P NMR spectra of suspensions of MDA-MB-468 cells in the growth medium (2 ml). Bottom, cells incubated for 24 h with 10 μM gossypol (180 million cells); top, control (210 million cells). Peak assignments as in Fig. 3.

Fig. 6. 31P NMR spectra of suspensions of cells incubated for 24 h with 10 μM rhodamine 123. Bottom, MCF-7 WT (190 million cells in 2 ml medium); top, MCF-7 ADR (220 million cells in 2 ml medium). Peak assignments as in Fig. 3. Control spectra were similar to those shown in Figs. 3 and 4.
were monitored during 25 h of incubation with 10 μM gossypol or 10 μM rhodamine. Since under normal circumstances cancer cells produce a significant proportion of their energy from glutamine (39), we performed the experiments with normal IMEM which contains 2 mM glutamine, as well as with medium without glutamine, in order to force the cells to base their energy production exclusively on glucose.

The results (Figs. 7 and 8) demonstrate great differences in the effect of gossypol and rhodamine on glucose consumption and lactate production between MCF-7 WT and MCF-7 ADR cells. With the ADR cells, 10 μM gossypol and 10 μM rhodamine have hardly any effect when the normal medium was used. In the absence of glutamine, when the effect of the drugs on glucose metabolism may be expected to be stronger, gossypol caused a slight increase of glucose consumption and a corresponding increase of lactate production (Fig. 7), whereas rhodamine, which is not toxic to the resistant cells at this concentration level (Table 1), had no effect.

In the case of the MCF-7 WT cells, gossypol and rhodamine had pronounced effects on glucose metabolism in each of the two types of media used (Fig. 8). Both drugs increased glucose consumption and lactate production; the effect of rhodamine was stronger and instantaneous, whereas the effect of gossypol was apparent only after several h of incubation (Fig. 8). As in the case of the resistant cells, the metabolism in the glucose-

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**Fig. 7.** Changes in glucose (-----) and lactate (-----) levels in medium during incubation of MCF-7 ADR cells with 10 μM gossypol and 10 μM rhodamine 123. Top, normal IMEM; bottom, IMEM without glutamine. Data correspond to 150-cm² flasks containing 50 ml of medium and about 35 million cells. Bars, SD.

**Fig. 8.** Changes in glucose (-----) and lactate (-----) levels in medium during incubation of MCF-7 WT cells with 10 μM gossypol and 10 μM rhodamine 123. Top, normal IMEM; bottom, IMEM without glutamine. Data correspond to 150-cm² flasks containing 50 ml of medium and about 35 million cells. Bars, SD.

**Fig. 9.** Ratios between lactate produced and glucose consumed after 14 h of incubation of MCF-7 WT cells with 10 μM gossypol and 10 μM rhodamine 123. Data from Fig. 8. Bars, SD.

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free medium was considerably slower, except for the rate of lactate production in the presence of rhodamine, which was practically the same in both media in spite of a decreased glucose uptake in the absence of glutamine. It follows that in the latter case the cells are considerably more glycolytic, pro-
ducting more lactate from a given amount of glucose. This is illustrated in Fig. 9, which shows ratios between the amount of lactate produced and glucose consumed after 14 h of incubation. At this point the amounts of glucose consumed and lactate produced during the experiment are already high enough to be measured accurately, and the glucose level in the medium is still sufficiently high; otherwise changes in the glucose utilization pattern due to glucose depletion (Crabtree effect) (39, 40) and irrespective of the drugs could interfere. The ratio was affected only slightly by gossypol, whereas rhodamine had a pronounced effect, especially in the absence of glutamine (Fig. 9).

DISCUSSION

Cancer cells that develop resistance to one drug tend to exhibit resistance against other drugs with different mechanisms of action (41, 42). This multiple drug resistance may be considered as a response of cancer cells to exploit their general detoxification ability to a much greater extent than in drug-sensitive cells. A key element in the detoxification strategy is expression of the P-170 membrane glycoprotein that is involved in drug export from the cell (42), and transfection of wild type cancer cells with the gene encoding P-170 (the MDR1 gene) is sufficient to bring about the multiple drug resistance phenotype (30, 31). A challenge to pharmacologists is to develop drugs that either are not subject to the generality of multiple drug resistance or are capable of turning the resistance mechanisms off.

Unlike the majority of currently used anticancer drugs, which have the nucleus and DNA replication processes as the targets of action, gossypol and rhodamine 123 affect energy metabolism of the cells. Previous studies suggested that the two compounds have a very similar mode of action, and both are designated as antimitochondrial drugs (6, 43). However, the alleged antimitochondrial effect of gossypol was proposed mainly on the basis of the fact that gossypol inhibited mitochondrial uptake of rhodamine 123 (44), and additional data on the mechanism of action of gossypol on cancer cells are of pertinent interest.

Comparison of the toxicity profiles of gossypol and rhodamine obtained in this work showed considerable differences (Fig. 1); cell lines selected for resistance with various drugs exhibited several hundred-fold resistance towards rhodamine, but no resistance towards gossypol (Table 1). Only the A2780/CP70 cells, which were selected for the resistance against platinum drugs and which apparently did not develop the multiple drug resistance phenotype to any significant extent (32), were sensitive to rhodamine as well as to gossypol. The drug-sensitive DC-3F cells were surprisingly resistant to rhodamine but, again, not to gossypol (Table 1). The ability of multiple drug-resistant cells to deal successfully with rhodamine 123 has been observed before (45), but the sensitivity of such cell lines to gossypol has not been observed prior to this work.

Also cells made multidrug resistant by retroviral transfection with the MDR1 gene (MDR MCF-7, BCA adr-19, FEMX 4A P, MDR-MDCK) were strongly resistant to rhodamine, but the resistance to gossypol was at most 2-fold (Table 1). The resistance towards rhodamine thus appears to be largely related to the operation of the P-170 pump. On the other hand, the sensitivity of the resistant cells to gossypol, in particular of the MDR1-transfected cells, either may mean that gossypol is merely not a substrate for P-170 or may be a manifestation of deactivation of P-170 by gossypol, e.g., by covalent linking as observed with other proteins (10); the distinction between these possibilities has yet to be made.

Experiments with glucose metabolism in the MCF-7 cells also showed considerable differences between the effects of rhodamine and gossypol (Figs. 7-9). Significantly, the presence of rhodamine markedly increased the ratio between lactate produced and glucose consumed in MCF-7 WT cells as compared with controls (Fig. 9). This effect was especially evident with the glutamine-free medium, where glucose was the sole energy source available to the cells. It is known that glycolysis alone can provide tumor cells with sufficient energy and metabolites for survival and growth (39). The high lactate production in the glutamine-free medium in the presence of rhodamine (MCF-7 WT cells) is especially noteworthy because in this case essentially all lactate must originate from glucose, unlike in the normal medium, where glutaminolysis presumably contributes to the lactate formed (39). In the presence of gossypol, as well as in the case of the controls, the lack of glutamine caused a decrease in relative lactate production (Fig. 9). Enhancement of aerobic glucose utilization under reduced glutamine concentrations is well known (39), but the observed decrease in the overall glucose consumption in the glutamine-free medium in the absence of the drugs (Fig. 8) is somewhat unexpected and may be related to the fact that high carbon flux from glucose and glutamine under normal conditions exceeds energy requirements and is related to activation and regulation of biosynthetic pathways (46).

The inhibition of oxidative phosphorylation and the forced dependence of the cells on glycolysis are thus presumably the essence of the toxic effect of rhodamine 123. This leads in cultured cells to accelerated glucose depletion and acidification of the medium, resulting in cell starvation and intoxication. The toxic effect of rhodamine may therefore be essentially an in vitro effect, and tissues continuously perfused and supplied with new nutrients in the body may be less susceptible to the effects of this drug. Although gossypol also increased glucose utilization, this took place to a lesser extent, and gossypol did not change the (lactate produced)/(glucose consumed) ratio (Fig. 9). This demonstrates that no significant shift in the relative importance of oxidative phosphorylation and glycolysis took place.

We also applied 31P NMR spectroscopy methods that have been developed to monitor cancer cell metabolism (36, 37) in order to evaluate effects of gossypol on drug-sensitive and drug-resistant cancer cells and to compare them with those of rhodamine 123. The decrease of ATP levels observed in 31P NMR spectra of cells treated with both drugs (except for the MCF-7 ADR cells treated with rhodamine, which is not harmful to these cells) is an immediate indicator of their toxicity. However, the response of other phosphorus-containing metabolites to gossypol and rhodamine was sharply dissimilar (Figs. 3–6; Tables 2 and 3). While rhodamine caused little change of peaks other than ATP, the increase of the level of pyridine nucleotides and the drop in the levels of phosphomonoesters and phosphodiesters indicates the sites of effects caused by gossypol.

Gossypol has long been linked to action of NAD(P)-dependent enzymes, particularly lactate dehydrogenase (1, 2), and tumor cells enriched in cathodal lactate dehydrogenase isozymes were shown to be especially sensitive to gossypol (6). Recently, gossypol was shown to interfere with the metabolism of 6-aminonicotinamide to 6-amino-NAD (43). The observed increase of cytoplasmic concentration of pyridine nucleotides...
(Table 2) may be due to interference with redox processes involving NAD(H) and NADP(H) or to direct interaction with enzymes that utilize them, resulting in a release of these coenzymes from macromolecular sites.

On the other hand, changes in the levels of phosphomono- and diesters, notably GPC, presumably reflect the action of gossypol on lipid membranes. It has been shown previously by other techniques that gossypol decreases the fluidity of lipid membranes in vitro (4, 14, 15). Since GPC was suggested to modulate membrane fluidity (47), the decreased cytoplasmic level of GPC (Table 2) may reflect this effect. The increase of membrane rigidity and possibly alteration of membrane proteins due to Schif base formation (10) presumably accounts for the difficulties in keeping the cells in the agarose threads during the previously mentioned perfusion experiments with gossypol. The alteration of plasma membranes may also affect the function of P-170 and play a role in the sensitivity of multidrug-resistant cells to gossypol.

Although 31P NMR spectra of cells influenced by gossypol were recorded previously (5, 6, 43), the observed effect on the spectra was restricted to a nondifferentiated decrease of the peaks, being merely indicative of toxicity and cell death. In our work, more subtle and differentiated spectral changes were observed, involving a decrease of some signals and increase of others. This emphasizes the utility of the 31P NMR method for studying mechanisms of action of antitumor drugs.

All experiments performed in this work showed differences between the effects of gossypol and rhodamine 123. These differences are not unexpected taking into account the cationic character of gossypol, being thus negatively charged at physiological pH. The antimitochondrial effect of rhodamine 123 is intimately associated with the positive charge; uncharged analogues do not accumulate in the mitochondria (49, 50). Because of the negative potential of mitochondrial membranes and the anionic character of gossypol, it is unlikely that it can mimic the action of rhodamine 123, but it can change the distribution of rhodamine in the cells by affecting membrane potentials, alteration of permeability parameters, or interaction between the charges. The action of gossypol on cancer cells is likely to be complex and involve multiple activities at various sites. These appear primarily to include an interaction with cellular processes involving NAD(H) and NADP(H) and, by virtue of the lipophilicity of gossypol, effects on lipid membranes.

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REFERENCES


Action of Gossypol and Rhodamine 123 on Wild Type and Multidrug-resistant MCF-7 Human Breast Cancer Cells: $^{31}$P Nuclear Magnetic Resonance and Toxicity Studies

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