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Research Article

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Lactic Dehydrogenase Isozymes, ^{31}P Magnetic Resonance Spectroscopy, and In Vitro Antimitochondrial Tumor Toxicity with Gossypol and Rhodamine-123

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Abstract

Three compounds that share specific antimitochondrial properties are gossypol, rhodamine-123, and lonidamine. We compare the antiproliferative activities of these drugs against six human cell lines derived from breast (T47-D), pancreas (MiaPaCa, RWP-2), prostate (DU-145), colon (HCT-8), and cervix (HeLa) carcinomas. Tumor cells enriched in cathodal LDH isozymes (LDH₄ and LDH₅) are significantly more sensitive to gossypol and rhodamine-123. When compared for ability to inhibit growth of human marrow in soft agar, 10 μM gossypol shows little effect on colony formation whereas 10 μM rhodamine-123 completely prevents stem cell growth, suggesting that gossypol may have the most favorable therapeutic index. Within 24 h of drug administration, there is a relative increase in intracellular inorganic phosphate pools and a marked decline in soluble high-energy phosphates in sensitive tumor cells, as measured by ^{31}P magnetic resonance spectroscopy. These studies suggest that specific antimitochondrial agents might be selectively administered on the basis of tumor LDH isozyme content and noninvasively monitored for antiproliferative activity by ^{31}P spectroscopy.

Introduction

Cancer cells, like normal cells, derive their cellular energy from ATP generated either by mitochondrial respiration and oxidative phosphorylation or by glycolysis and the degradation of simple carbohydrates to lactic acid. In 1930 Warburg first described the ability of normal cells to reduce glycolysis and lactic acid production in the presence of oxygen (1). Known as the Pasteur effect, this important form of multienzyme control is believed to originate from mitochondrial signals. Warburg also noted that cancer cells lack this regulatory control over lactic acid production and he postulated that the neoplastic process is induced by some impairment in mitochondrial respiration (2).

Although few investigators currently believe that malignant transformation originates from within mitochondria, Warburg's hypothesis generated great interest in the study of mitochondrial function. Presently, a number of associated features are known

to differentiate normal from malignant mitochondria, and these are reviewed by Pederson (3). Among these distinguishing features is a frequently observed isozyme shift by oxidoreductases such as lactic dehydrogenase, (LDH,¹ L-lactate: NAD oxidoreductase, EC 1.1.1.27) (3, 4). Such isozyme shifts favor enhanced ATP production under anaerobic conditions and reduced feedback regulation over glycolysis. For example, increased content of the cathodal isozyme, LDH₅, is observed in both sera and tumor samples of patients with advanced epithelial neoplasms (5–8). Another feature of malignant transformation is the augmented transmembrane potential within malignant mitochondria. This results in increased mitochondrial retention of lipophilic cationic dyes such as rhodamine-123 (9).

Recently, investigators have theorized that the differences between normal and malignant mitochondria might be exploited in the development of mitochondrial-specific antitumor agents (10). Three unique compounds that might now be classified as antimitochondrial agents include the supravital dye, rhodamine-123, the phenolic cotton seed (*Gossypium*) extract, gossypol, and the indazole carboxylic acid, lonidamine. These compounds are shown in Fig. 1.

The antitumor potential of rhodamine-123 was recognized shortly after its identification as a fluorescent mitochondrial dye (11–13). Gossypol and lonidamine were initially developed as antispermatogenic compounds; both are currently being tested for antitumor activity (14, 15). These three agents share common dose-dependent properties: enhanced accumulation into tumor mitochondria, inhibition of oxidoreductases and uncoupling of mitochondrial respiration, reduction in intracellular oxygen consumption and lactic acid production, and swelling of mitochondria followed by cytoplasmic leakage and decreased cellular synthesis of macromolecules.

Preliminary results indicate that the antitumor toxicity of these agents is very selective (13, 14, 16), perhaps explaining the failure of early screening procedures to detect their potential chemotherapeutic application (17). In this report we identify a simple assay that may be helpful in identifying tumors most sensitive to antimitochondrial agents. We also compare the antitumor activity of these three drugs with their ability to inhibit clonogenic growth of normal human bone marrow stem cells. In addition, we demonstrate the potential ability of ^{31}P magnetic resonance spectroscopy to provide a noninvasive means of monitoring the biochemical changes induced by a toxic dose of either gossypol or rhodamine-123.

Methods

Growth inhibition of human tumor cell lines and bone marrow stem cells. The well-established monolayer human carcinoma cell lines T47-D

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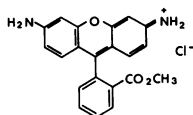
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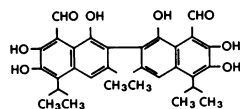
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1. *Abbreviations used in this paper:* LDH, lactic dehydrogenase; Pi, inorganic phosphate.

Rhodamine 123



Gossypol



Lonidamine

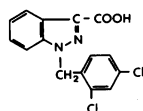


Figure 1. Three structurally dissimilar compounds that share specific antimitochondrial properties.

(breast), MiaPaCa (pancreas), RWP-2 (pancreas), DU-145 (prostate), HCT-8 (colorectal), and HeLa (cervical) are maintained as stock cultures in 5% CO₂ incubators at 37°C, using RPMI 1640 media (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; HyClone Laboratories, Logan, UT). The estrogen receptor-positive T47-D cells are also supplemented with 0.2 IU/ml insulin (Sigma Chemical Co., St. Louis, MO). Growth characteristics of these cells have been described previously (18–20). All stock cultures are passaged weekly by trypsinization up to 25 times before being replaced by frozen stocks.

For assessment of tumor cell toxicity or growth inhibition, cells (5 × 10⁴) are plated on day 0 into triplicate 25-cm² sterile plastic flasks (Costar, Cambridge, MA) and allowed to attach for 24 h before drug addition on day 1. The antimitochondrial drugs are added from ethanol-containing stock solutions to a final ethanol concentration ≤ 0.1% (vol/vol). Control flasks contain an equivalent ethanol concentration that does not inhibit culture growth. Gossypol and rhodamine-123 were obtained from Sigma Chemical Co. Lonidamine was generously provided by Dr. B. Silvestrini, Instituto Di Ricerca F. Angelini, Rome. Exposure to these agents is continuous until flasks are harvested on day 6, while control culture growth is still logarithmic. Harvested cells are counted on a model ZB1 counter (Coulter Electronics, Inc., Hialeah, FL), and results of growth inhibition studies are reported as either the mean (±SD) cell number, the percent of control cells counted, or IC₅₀, the concentration of drug resulting in 50% inhibition of control growth.

To culture bone marrow stem cells, we inject freshly aspirated normal bone marrow from consenting patients into sterile tubes containing preservative-free heparin (1,000 IU). The marrow suspension (7 ml) is layered onto 3 ml of lymphocyte separation medium (Bionetics Laboratory Products, Kensington, MD), centrifuged, and the stem cell layer is carefully removed. Remaining erythrocytes are removed by hypotonic shock, and the cells are then washed twice with McCoy's medium 5A (Gibco Laboratories) containing 10% FCS. These isolated bone marrow stem cells are cloned with a standard bilayer-agar methodology. Cells are suspended in 0.3% Bacto-agar in medium supplemented with 20% FCS. This mixture (1 ml containing 10⁵ cells) is transferred to replicate 35-mm plastic wells containing a 1-ml feeder layer of 0.5% agar in medium containing 20% serum, with or without drug. After the agar is set, 0.1 ml of conditioned medium (GCT; Gibco Laboratories) is added to cover the surface of the culture. Cultures are then incubated at 37°C in a 5% CO₂-humidified atmosphere with no additional feeding, and colony formation is microscopically quantitated at 9–12 d after plating. The cloning efficiency of untreated marrow cells processed in this manner is ~ 0.1%.

LDH isozyme activity. Harvested cell (10⁷) pellets are rinsed and suspended in cold phosphate-buffered saline (PBS) (5 ml, 4°C), sonicated (200 W × 5 s × 4) in an ice bath, centrifuged at ~ 10,000 g for 5 min, and the supernatants are collected. Total LDH enzyme and isozyme activity is measured from ice-cold diluted aliquots of these supernatants within 30 min of lysate preparation with a routine clinical assay that we have previously described (21). The agarose gel electrophoresis and flu-

orometric detection of LDH isozymes is performed on a Corning Medical and Scientific (Palo Alto, CA) system using ~ 4,000 U/l of LDH-containing lysate, with AMP lactate (pH 9.0) and NAD as substrates. Gels are photographed and densitometrically scanned under UV illumination for detection and quantitation of the five tetrameric LDH isozymes, as well as the unique germ cell-specific isozyme LDH_{C4}, also known as LDH-X (21). Human serum and lysate from sperm-containing ejaculate are used as assay standards, demonstrated in Fig. 2. Scanned peaks of isozyme activity are quantitated as percent total LDH activity with a Beckman Model R-110 densitometer and integrator. For regression analysis, the percent of cathodal isozyme activity is calculated as either the combined percentage of LDH₄ + LDH₅ activity, or by determining the total percent of the LDH-M monomer using the formula (8): % LDH-M = % LDH₅ + (0.75 × % LDH₄) + (0.5 × % LDH₃) + (0.25 × % LDH₂). The percent anodal isozyme activity is calculated as either the combined percentage of LDH₁ + LDH₂ activity, or by determining the total percent of the LDH-H monomer (100 - % LDH-M).

³¹P magnetic resonance spectroscopy. All spectroscopy experiments are carried out on a system consisting of a 5.6-tesla superconducting magnet using a ³¹P resonance frequency of 97.59 MHz, a Nicolet computer (NIC 1180), and customized transmitter, receiver, filter, and preamplifiers. A typical spectrum is obtained using 60°-radiofrequency pulses over a 10-KHz sweep width, a recycle time of 2.2 s, quadrature detection over 4K data points in ~ 30 min, and exponential line broadening of 30 Hz. Spectra are referenced to an internal capillary containing methylenediphosphonic acid (0.5 M) in a Tris buffer (pH 7.4) at a run temperature of 4°C. Control or drug-treated cells (10⁶) that remain intact and attached as monolayers are harvested by trypsinization, rinsed with 0.9% NaCl containing 20 mM Tris HCl (pH 7.5) and 3 mM MgCl₂, loosely pelleted and placed into a 10-mm diameter glass tube within the homogeneous region of the magnetic field. Control studies have shown that neither cell viability (by trypan blue dye exclusion or plating efficiency) nor phosphorus spectra are significantly altered if spectroscopy is completed within 90 min of culture harvest under these conditions. Beyond 120 min, however, there is a noticeable decline in the β-ATP spectral peak intensity and a concomitant increase in the inorganic phosphate (Pi) peak intensity. Spectral shifts are identified with respect to phosphocreatine (0 ppm) and conform with previous reports (22–24). Intracellular pH is determined from the Pi shift and calculated from a standard curve (25).

Statistical analysis. Pearson's linear regression and the nonparametric Spearman's rank correlation techniques are used to analyze the relationship between LDH isozymes and toxicity to gossypol and rhodamine-

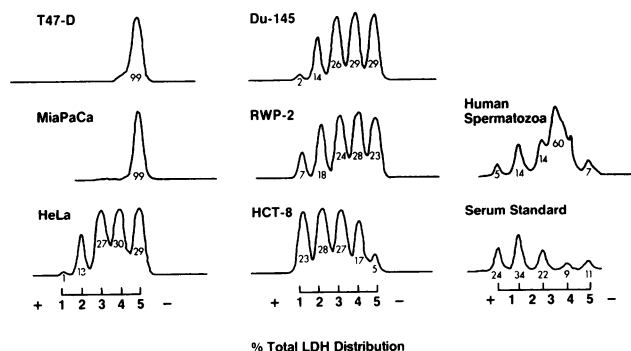


Figure 2. Quantitation of tumor cell LDH isozymes. Tumor cell extracts containing LDH are prepared, electrophoresed and scanned for isozyme activity as described in Fig. 5. Peaks of isozyme activity are labelled from the most cathodal (-) species, LDH₅, to the most anodal (+) species, LDH₁, corresponding to a known serum standard. Integrated peak area yields a value of percent total LDH activity for each isozyme, as shown. Human spermatozoa contain an abundance (60%) of the unique isozyme, LDH-X.

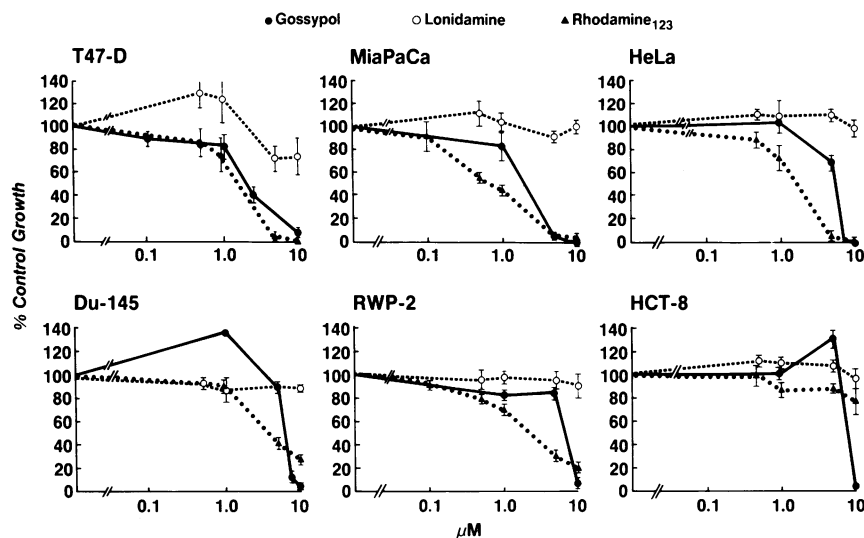


Figure 3. Dose-dependent in vitro growth inhibition of human carcinoma cell lines by gossypol, lonidamine, and rhodamine-123. Cells (5×10^4) are plated on day 0 into triplicate flasks, the indicated concentration of drug is added on day 1, and total cell number per flask is determined on day 6. Results from two separate experiments are pooled, averaged, and expressed as percent control growth (\pm SD). The mean total cell count achieved in control flasks is as follows: T47-D (1.6×10^6), MiaPaCa (1.6×10^6), HeLa (3.2×10^6), DU-145 (0.4×10^6), RWP-2 (0.3×10^6), HCT-8 (0.7×10^6).

123. The significance level (P value) is determined with a two-tailed t test with $n-2$ degrees of freedom.

Results

Fig. 3 compares the in vitro growth inhibiting potency of rhodamine-123, gossypol, and lonidamine against human breast carcinoma cells, T47-D, two different pancreatic carcinomas, MiaPaCa and RWP-2, the prostate carcinoma, DU-145, the colorectal carcinoma, HCT-8, and the cervical carcinoma, HeLa. Lonidamine at doses of up to $10 \mu\text{M}$ shows minimal ($\leq 30\%$) activity against T47-D cells but virtually no activity against the other cell lines. At doses $\leq 5 \mu\text{M}$, rhodamine-123 appears slightly more potent than gossypol, with T47-D, MiaPaCa, and HeLa cells demonstrating the greatest sensitivity to both drugs, respectively. Although these three cell lines proliferate more rapidly than the others in culture, overall tumor cell sensitivity to these agents does not correlate well with culture doubling times. For example HeLa cells, inhibited 30% by $5 \mu\text{M}$ gossypol, grow about twice as fast as MiaPaCa cells, which are inhibited more than 90% by the same drug dose. Other experiments established the dependence of gossypol potency on serum content. MiaPaCa cells grow equally well in 5%, 10%, or 15% FCS; but the dose of gossypol necessary to inhibit growth by 50% under these conditions is $2.3 \mu\text{M}$, $2.7 \mu\text{M}$, and $3.5 \mu\text{M}$, respectively.

Drug sensitivity of human marrow stem cell growth in soft agar is illustrated in Fig. 4. Repetitive experiments yielded similar results, with rhodamine-123 appearing most toxic. Use of drug concentrations up to $100 \mu\text{M}$ showed that $10 \mu\text{M}$ gossypol barely affects marrow growth, $15 \mu\text{M}$ inhibits growth 50%, and $50 \mu\text{M}$ gossypol abolishes all stem cell growth. In comparison, $5 \mu\text{M}$ rhodamine-123 inhibits marrow growth by 50% and $10 \mu\text{M}$ abolishes all growth. Lonidamine, on the other hand, marginally reduces marrow growth at concentrations above $5 \mu\text{M}$, and even with doses of up to $100 \mu\text{M}$ nearly 75% of control growth is still observed.

Table I relates the gossypol and rhodamine-123 bone marrow and tumor cytotoxicity results in terms of IC_{50} (\pm SD) values to compare the in vitro therapeutic indices of these two drugs. While slightly more potent at inhibiting tumor cell growth, rhodamine-123 appears about five-fold more myelosuppressive, thus reducing its potential therapeutic index.

LDH isozymes are identified by agarose electrophoresis, as illustrated for five of the tumor cell lines in Fig. 5. The percent relative activities of these isozymes for each of the carcinoma lines remains constant under all culture conditions, but varies considerably between cell lines even of the same tumor type. Fig. 2 gives the calculated distribution of isozyme activities beneath the densitometric profiles and compares the individual tumor cell lines with a serum standard and an extract from human spermatozoa, which contain an abundance (60%) of the unique LDH-X isozyme. The two pancreatic adenocarcinomas,

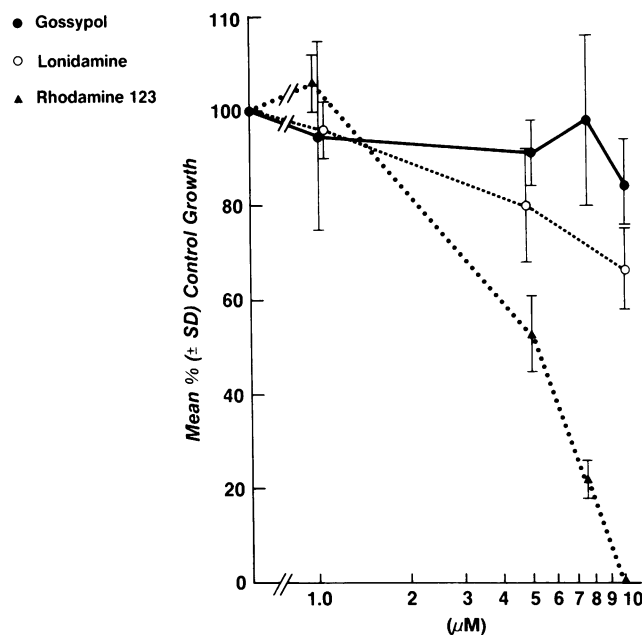


Figure 4. Dose-dependent inhibition of human bone marrow clonogenic growth in soft agar. A fractionated suspension of nucleated marrow stem cells (1×10^5) are cloned in the presence or absence of indicated concentrations of gossypol, lonidamine, or rhodamine-123 with a standard bilayer-agar technique. Colonies of 15 or more cells in replicate 35-mm wells are microscopically counted 9–12 d after plating. The results of two separate experiments are averaged and recorded as the mean (\pm SD) percent control colony growth. Control growth (100%) resulted in an average value of 52 ± 3 marrow colonies per well.

Table I. IC_{50} Values* for Gossypol and Rhodamine-123 against Human Tumor Cell Lines and Bone Marrow Stem Cells

	Gossypol	Rhodamine-123
MiaPaCa	2.0 (± 0.5)	0.7 (± 0.2)
T47-D	2.0 (± 0.3)	1.9 (± 0.4)
HeLa	5.6 (± 0.2)	1.7 (± 0.4)
RWP-2	6.8 (± 0.4)	2.2 (± 0.3)
DU-145	6.1 (± 0.2)	3.7 (± 0.8)
HCT-8	7.8 (± 0.4)	>10.0
Bone marrow	15.0 (± 2.0)	5.2 (± 0.6)

* IC_{50} values are micromolar concentrations inhibiting in vitro growth by 50% (\pm SD).

MiaPaCa and RWP-2, have markedly different isozyme profiles. The MiaPaCa cells, which have been shown to contain high-affinity receptor for estradiol much like the T47-D cells (20), are also similar to these breast cancer cells in their expression of a single cathodal isozyme, LDH₅. Normal human marrow was found to be very heterogeneous in its LDH isozyme content. Marrow cells isolated from above, within, and below the nucleated cell fraction containing clonogenic stem cells showed LDH₁ + LDH₂ content ranging from 32 to 52% of total LDH activity, and LDH₄ + LDH₅ content varying from 44 to 33% of total LDH. It was not feasible to analyze LDH isozymes from the small number of actual clonogenic stem cells.

Because the T47-D and MiaPaCa carcinomas appeared most sensitive to gossypol and rhodamine-123 and most similar in their isozyme profiles, we attempted to correlate the tabulated IC_{50} values for these two drugs from Table I, and the percent anodal or cathodal isozyme values obtained from Fig. 2. As can be seen in Fig. 6 A, a positive linear regression exists between drug resistance (increased IC_{50}) and anodal isozymes (LDH₁ + LDH₂) for both antimetabolites. Likewise, Fig. 6 B shows that increased drug sensitivity (decreased IC_{50}) appears to correlate with cathodal isozyme (LDH₄ + LDH₅) content. The results are similar when total percent LDH-H or LDH-M monomers are calculated and substituted into the regression analyses in place of percent anodal or cathodal isozymes. These

data were also analyzed by rank correlation due to uncertainty in the linear regression analysis for rhodamine-123. By rank analysis, cathodal isozyme content also correlated significantly with both gossypol ($r = -0.96$; $P < 0.05$) and rhodamine-123 ($r = -0.89$; $P < 0.05$) IC_{50} values. When the outlying HCT-8 data values are omitted from this analysis, the rank correlation coefficients are only slightly reduced, confirming the validity of this correlation.

Although growth-inhibiting concentrations of gossypol and rhodamine-123 do not significantly alter cell morphology or surface attachment of these cultures within the first 24 h of drug treatment, we examined the early biochemical effects of such treatment with ³¹P magnetic resonance spectroscopy. Figs. 7 and 8 compare the phosphorus spectrum of control MiaPaCa and T47-D cell cultures, respectively, with cultures treated either by gossypol (5 μ M) or rhodamine-123 (5 μ M) for 1 and 24 h before harvest. Seven primary peaks of ³¹P signal intensity can be identified in the untreated cells, consistent with observations in other cultured cells and in vivo models (22-24). These peaks correspond to soluble intracellular forms of phosphomonoesters, Pi, phosphodiester, phosphocreatine, γ -ATP + β -ADP, α -ATP + α -ADP + NAD/NADH, and β -ATP. An intracellular pH of nearly 7.0 is determined from the spectral shift of Pi. After 1 h of gossypol or rhodamine-123 treatment, a marked relative increase in Pi content is apparent in both cell lines, with no detectable change in intracellular pH. After 24 h of treatment, Pi is present as the most dominant soluble phosphorus species, with almost undetectable amounts of soluble high energy phosphates remaining in MiaPaCa or T47-D cells. Growth inhibiting doses of selected antimetabolites (e.g., methotrexate, 5-fluorouracil) or alkylating agents (e.g., cis-platin) did not produce comparable patterns of ³¹P spectral change in these or other tumor cell lines (data not shown).

Discussion

Tumor mitochondria differ functionally from normal mitochondria by sustaining high rates of glycolysis and lactic acid production with exposure to either high or low oxygen tension. Thus, they provide tumor cells with a relative advantage in energy (ATP) production, especially under anaerobic conditions. Both normal and malignant cells depend on NAD(P) pools to support

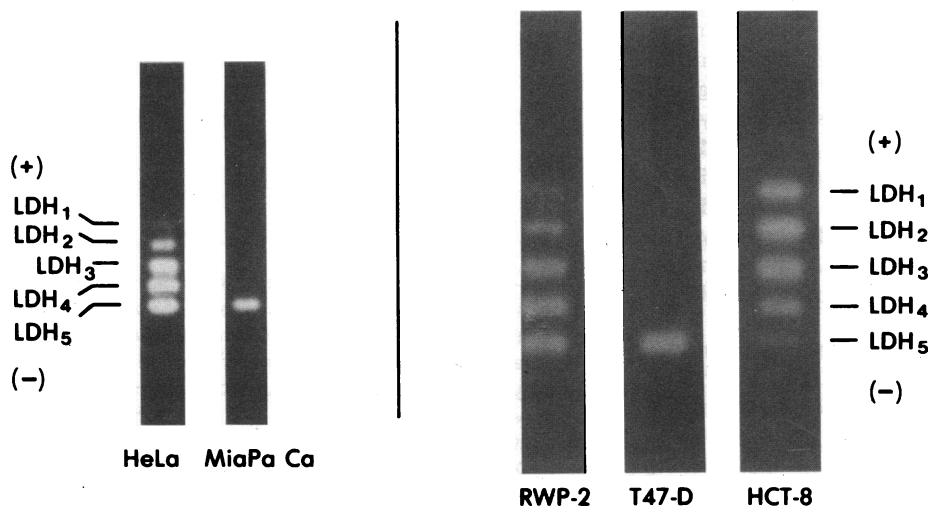


Figure 5. Agarose electrophoresis to fractionate LDH isozymes. Untreated tumor cells (10^7) are harvested, sonicated, and centrifuged to obtain supernatants containing LDH. $\sim 4,000$ U/l of LDH containing extracts are electrophoresed and detected by fluorometric reaction with an NAD-containing substrate mixture. Gels are photographed and scanned under ultraviolet illumination to detect and quantitate the five tetrameric isozyme species identified above.

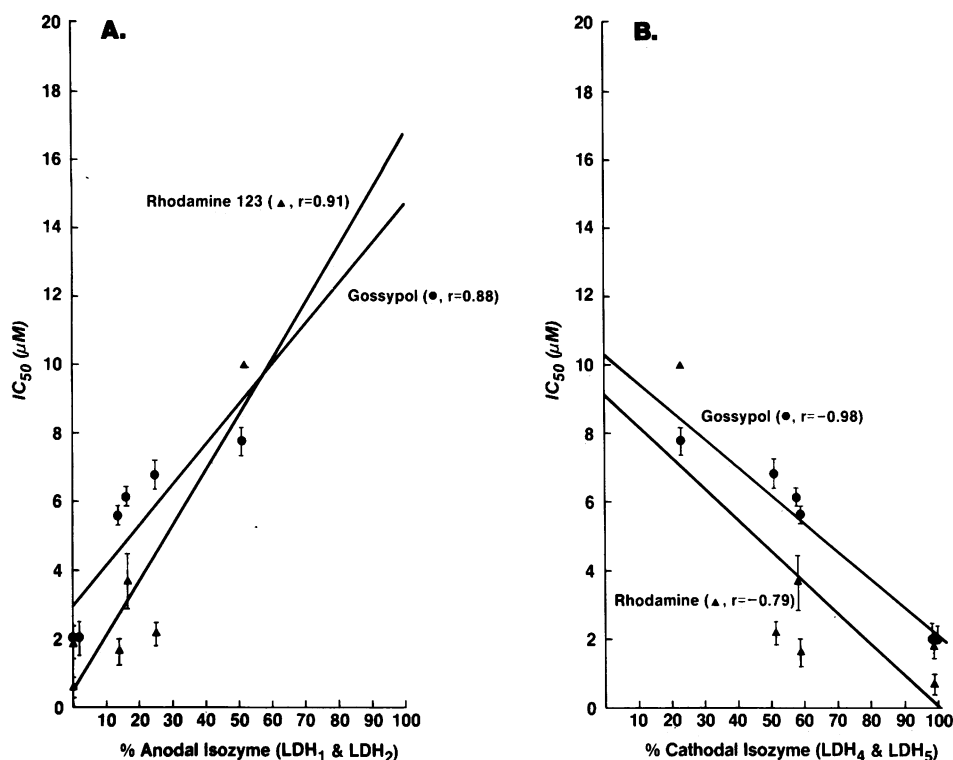


Figure 6. Linear regression analyses of gossypol and rhodamine-123 IC_{50} values and percent anodal (A) or cathodal (B) isozyme content. Each data point represents a given tumor cell line, its 50% inhibitory concentration (IC_{50} , $\pm SD$) of either gossypol (solid circle) or rhodamine-123 (solid triangle), and its percent anodal LDH₁ + LDH₂ (A), or percent cathodal LDH₄ + LDH₅ (B) isozyme content. Correlation coefficients (r) are indicated for each regression line; all coefficients are significant at $P < 0.05$ except rhodamine-123 in (B), which is significant at $P < 0.10$. When total percent LDH-H or LDH-M monomers are calculated (8) and substituted into the linear regression analyses in place of percent anodal or cathodal isozymes, respectively, the following values are obtained for A (%LDH-H): gossypol, $r = 0.98$; rhodamine-123, $r = 0.79$; and B (%LDH-M): gossypol, $r = -0.98$; rhodamine-123, $r = -0.79$.

glycolysis, mitochondrial respiration, synthesis of pentose-phosphates, and metabolism of lipids and steroids. The only extramitochondrial enzyme available to fill this demand on NAD pools is LDH. By shifting to more cathodal forms of LDH, malignant cells in a growing tumor mass can better adapt to an anaerobic milieu. The LDH-M subunit, stoichiometrically dominant in LDH₄ and LDH₅ isozymes, most efficiently converts pyruvate to lactate, generating NAD from NADH. This qualitative difference in energy metabolism between normal and malignant cells also represents a potential new target for selective chemotherapy.

The condensed (state III) form of mitochondria within epithelial neoplasms appears susceptible to quinonelike compounds that can uncouple electron transport and inhibit oxidative phosphorylation (3). Lonidamine, rhodamine-123, and gossypol have been shown to preferentially accumulate within condensed tumor mitochondria and lead to inhibition of tumor cell growth (12–14, 26, 27). While the underlying molecular mechanisms

accounting for the antimitochondrial and growth inhibiting effects of these agents are not completely understood, a variety of NAD-dependent enzymes including LDH are known to be directly inhibited by each of these drugs. For example, within the first moments of a cell's exposure to gossypol, mitochondrial uptake with fluorescent dye is reduced and LDH is irreversibly inactivated (28, 29). The cathodal isozymes LDH₅ and LDH-X are known to be most susceptible to gossypol inhibition, possibly by competition for their NADH-binding sites (28). Other investigators have suggested that enzyme and cell growth inhibition are mediated by gossypol chelation of Fe^{+2} (30, 31).

We have extended these earlier findings by comparing human tumor and bone marrow stem cell toxicity with gossypol, rhodamine-123, and lonidamine, and correlating this cytotoxicity (IC_{50} values) with LDH isozyme profiles in the tumor cells. Lonidamine at doses approaching 10 μM inhibits human marrow stem cell growth $\sim 25\%$, and produces $< 30\%$ growth inhibition of all the human tumor cell lines tested. Numerous clinical stud-

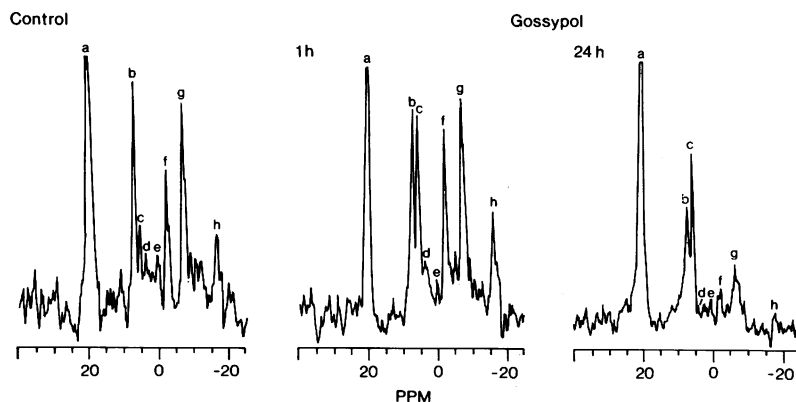


Figure 7. ^{31}P magnetic resonance spectroscopy of control and 5 μM gossypol-treated MiaPaCa cells. Control and treated (1 h, 24 h) monolayer cultures are harvested into a buffered saline solution at 4°C, and loosely pelleted cells are placed into a glass tube within the 5.6-tesla magnetic field. Spectra are obtained at 4°C, completed within 90 min of cell harvest, and referenced to a capillary containing 0.5 M methylenediphosphonic acid (a). Intracellular pH is determined from the Pi shift (c) and a calibrated standard curve. The remaining peaks of phosphorus signal intensity are identified as intracellular phosphomonoesters (b), phosphodiester (d), phosphocreatine (e), γ -ATP + β -ADP (f), α -ATP + α -ADP + NAD/NADP (g), and β -ATP (h).

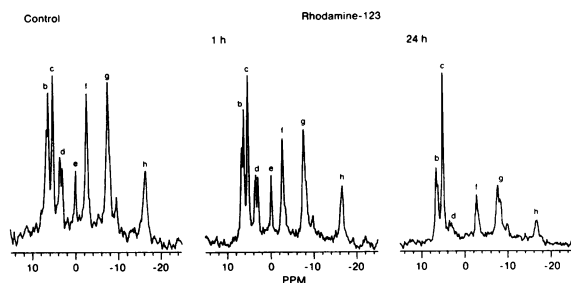


Figure 8. ^{31}P Magnetic resonance spectroscopy of control and rhodamine-123 ($5\ \mu\text{M}$) treated T47-D cells. Spectra are obtained on control and treated (1 h, 24 h) cells with phosphorus signal intensities identified as described in Fig. 7.

ies have indicated that lonidamine is not particularly myelo-suppressive; however, clinical antitumor responses are also rarely observed (14). In these studies, musculoskeletal side effects have been dose limiting and have prevented achieving in vivo drug levels $> 0.1\ \text{mM}$. Unfortunately, lonidamine concentrations exceeding $0.1\ \text{mM}$ appear to be necessary to inhibit tumor cell growth in vitro (32). Our in vitro results support the fact that lonidamine exhibits a very poor therapeutic index.

Among the three antimetabolic agents, rhodamine-123 is probably the most potent antiproliferative drug. However, a $10\text{-}\mu\text{M}$ dose of rhodamine-123, which inhibits growth of nearly every tumor line by $> 70\%$, also abolishes marrow stem cell growth. In contrast, a similar dose of gossypol appears nearly as potent as rhodamine-123 against the tumor cells but inhibits marrow stem cell growth by less than 20% . Thus, based on these in vitro results, the therapeutic index would appear to favor gossypol rather than rhodamine-123. Human use of gossypol has also been associated with musculoskeletal side effects (15). However, preliminary in vivo studies suggest that antitumor responses can be observed with doses of gossypol that are not associated with significant host toxicity (33).

The selectivity of gossypol's antitumor activity is confirmed by our results using a variety of common epithelial tumor lines not previously tested. The observed in vitro dependence of this drug's activity on serum content has also been recognized in nonmalignant cell systems (29). When experiments are controlled for serum content, the antitumor effectiveness of gossypol does not correlate with tumor type or with cellular growth rates in culture. While this lack of correlation with tumor growth rate is consistent with a prior report on gossypol cytotoxicity (16), it is an unusual observation for an S phase-specific drug (26) and suggests that other biochemical factors are more important in selection for gossypol's antiproliferative activity.

The significant correlations between tumor content of cathodal isozymes or LDH-M subunits and IC_{50} values for both gossypol and rhodamine-123 provide rationale for LDH isozymes to be used as a tumor marker for clinical evaluation of these drugs. It is known that LDH isozyme profiles are not specific for tumor types or grades of malignancy. We are, however, currently investigating the possibility that sex steroid-responsive tumors possess relatively greater amounts of LDH_4 and LDH_5 , since estrogens have been shown to stimulate uterine and mammary gland synthesis of these cathodal isozymes and inhibit synthesis of the anodal forms (34). Clinical specimens of solid tumor tissue may be readily assayed for LDH isozyme content, providing a rapid means of predicting sensitivity to antimetabolic

drial drugs like gossypol and rhodamine-123, or perhaps to other chemotherapeutic agents whose antitumor activity may be due in part to mitochondrial inhibition (35).

This study also illustrates the feasibility of ^{31}P magnetic resonance spectroscopy to detect prelethal changes in high-energy phosphate pools occurring in sensitive tumor cells within the first 24 h of exposure to gossypol or rhodamine-123. In preliminary studies with combined magnetic resonance proton imaging and ^{31}P spectroscopy of tumors implanted in rats, we have been able to document that these same spectroscopic perturbations occur in vivo within 3 h following a single injection of gossypol (36). These preclinical results provide reason to speculate that antimetabolic agents could ultimately be administered on the basis of an individual tumor's biochemical profile and clinically monitored for effectiveness within the first few hours of their administration.

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