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Laboratory investigation

In vitro and *in vivo* cytotoxicity of gossypol against central nervous system tumor cell lines

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Summary

Gossypol is a lipid soluble polyphenolic compound isolated from cotton seed oil which has been previously shown to have antiproliferative activity *in vitro* against a variety of human solid tumor cell lines. It has been extensively tested in clinical trials as a male contraceptive agent and found to be well tolerated. Its mechanism of action is thought to be inhibition of cellular energy metabolism. It inhibits glycolysis through inhibition of LDH isoenzyme type 5, and it inhibits mitochondrial oxidative phosphorylation and electron transport. We tested the *in vitro* antiproliferative effect of gossypol against four well characterized human glioma cell lines, HS 683, U373, U87 and U138, and one rat glioma cell line, C6, using the colorimetric Microculture Tetrazolium Assay (MTT). Cytotoxicity was found to be concentration and time dependent and increased with incubation times up to 8 days. The relative sensitivity of the glioma cell lines to gossypol at 48 hour incubation correlated with their respective LDH isoenzyme profiles, with the more sensitive cell lines expressing increased cathodal LDH isoenzymes (LDH 5). The *in vitro* cytotoxicity of gossypol to these CNS tumor lines was compared to the other non central nervous system solid tumor cell lines which had been previously reported as being sensitive to gossypol, including SW-13 (adrenal), MCF-7 (breast), T47-D (breast), and HeLa (cervical). Additional lines tested included SK-MEL-3 (melanoma), Colo 201 (colon) and BRW, a line established in our laboratory from a patient with a Primitive Neuroectodermal tumor. C6, HS 683, and BRW had similar IC50s as the sensitive solid tumor cell lines. U373, U87 and U138 had significantly less sensitivity at 48 hours. There was greater cytotoxicity and no significant differences in the IC50s between any of cell lines at 8 day incubations.

Additionally, we tested the cytotoxicity of gossypol against BRW *in vivo*, using the nude mouse xenograft model. Gossypol, given at a dose of 30 mg/kg per day five days a week for four weeks orally via gavage, was found to decrease the mean tumor weight of treated xenografts by more than 50% as compared to untreated xenografts. These findings suggest that gossypol has potential for further study as an agent for the treatment of primary CNS malignancies.

Introduction

Gliomas occur at a rate of approximately 5 per 100,000 per year and the incidence may be increasing [1]. Over half are high grade gliomas and these patients have an extremely poor prognosis. In a recent retrospective analysis of 285 patients with high grade glioma treated with cytoreductive surgery and radiotherapy, the median survival for all patients was only 35 weeks [2]. The prognosis for elderly patients with high grade glioma is even worse, with a 16 week median survival reported for patients over 60 treated with cytoreductive surgery and radiotherapy [3]. Currently available chemotherapy is estimated to decrease the tumor cell burden by only one log, which is two logs less than the

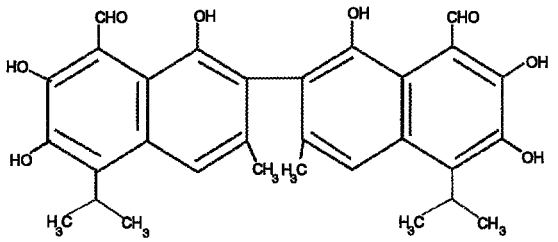

Fig. 1. The structure of gossypol.

decrease estimated to be necessary for curative potential when given in conjunction with radiotherapy and surgery [4]. It is generally accepted that nitrosoureas achieve response rates of approximately 50% in phase II studies in recurrent glioma [5]. Procarbazine used as a single agent has been found to have a response rate of approximately 27% using modern response criteria [6]. Combination chemotherapy with regimens such as Procarbazine, CCNU and Vincristine (PCV) may have higher response rates than single agent therapy with BCNU, but the advantage is slight [7]. A recent meta-analysis of adjuvant trials of alkylating agent based chemotherapy in high grade glioma revealed an overall modest survival benefit and increased time to progression for chemotherapy treated patients as opposed to controls. However, the benefit was not demonstrable in all subgroups and was limited to patient groups who had anaplastic astrocytoma [8]. Efforts to increase the efficacy of standard chemotherapy by escalating doses with autologous bone marrow rescue may improve response rates somewhat, but the applicability of this approach is somewhat limited by the inability of elderly patients to withstand these rigorous and expensive treatments. Clearly other strategies must be explored in the treatment of high grade glioma.

The development of new drugs with anti-glioma activity remains an important strategy. New agents which are relatively non-toxic and better tolerated by older patients, who make up the majority of the patients with high grade glioma, will be especially useful. Rosenblum *et al.* hypothesized that 'chemotherapy programs that are designed on the basis of metabolic capabilities of the target cells should be more successful than those that are not' [9]. The well described differences in the energy metabolism between normal brain and glioma cells are attractive targets for exploiting this strategy. These differences [10] are summarized as follows: Within the tumor cells there is: 1) A surplus of ATP; 2) A striking decrease in oxidative phosphorylation and activity of the Krebs cycle; 3) A striking increase in glycolysis even in the presence of oxygen (aerobic glycolysis), which allows for energy production in the tumor even under hypoxic conditions as long as an adequate supply of glucose is available; 4) An increase in glucose utilization; 5) An increase in the activity of the pentose phosphate shunt; 6) And a striking decrease in the number of mitochondria. While total LDH activity has been shown to be normal in glioma tissue, the isoenzyme pattern of glioma cells is shifted to the cathodal form of LDH: LDH5. This shift is correlated to the degree of anaplasia, with a shift being seen in 79% of grade III/IV tumors but only 14% of grade I/II tumors [11]. The cathodal form of LDH is generally associated with anaerobic glycolysis and is found normally in muscle tissue.

Gossypol, a polyphenolic, lipid soluble compound, isolated from cotton seed oil is a drug which could potentially exploit the differences in energy metabolism. Its structure is shown in Fig. 1. Gossypol has been shown to have antiproliferative activity *in vitro* against a number of tumor cell lines including melanoma [12], colon [12], cervical [13], adrenocortical [14] and breast [15]. It has been shown that gossypol uncouples electron transport, and inhibits oxidative phosphorylation, ATP production [16], and glycolysis [17]. Gossypol's inhibition of glycolysis is thought to be mediated by inhibition of Lactate dehydrogenase. Of the LDH isoenzymes, gossypol specifically inhibits LDH-5 and LDH-X (the form of LDH found in sperm) [18]. Studies by Benz *et al.* [13], and Tuszynski *et al.* [12], have shown that the *in vitro* antiproliferative effect of gossypol is correlated to the LDH profile of various cell lines tested, with lines shifted to LDH-5 being more sensitive.

Gossypol has been tested extensively in humans in clinical trials as a male contraceptive agent [19], as well in two clinical trials as an antineoplastic agent [20, 21], and has been found to be well tolerated. We undertook this study to determine the *in vitro* sensitivity of glioma cell lines to gossypol, and to

determine if the sensitivity correlated with the LDH profile of these cell lines. The MTT cytotoxicity assay was used to determine the *in vitro* sensitivity of the cell lines to gossypol. This technique has been reported to be highly reproducible [22] and has been used to screen various glioma cell lines with standard chemotherapeutic agents [23]. It has also been found to correlate to clinical sensitivity in glioma patients whose fresh tumors had been screened in short term culture with the MTT assay [24]. Additionally, we undertook a study using the nude mouse xenograft model [25] to define the *in vivo* cytotoxicity of gossypol against a human Primitive Neuroectodermal Tumor cell line, BRW, which had been established in our laboratory.

Methods

Cell lines and culture conditions

The establiehd human glioma cell lines U373 (HTB-17), U87 (HTB-14), HS 683 (HTB-138), U138 (HTB-16), and a rat glioma line C6 (CCL-107) were purchased from American Type Culture Collection (Rockville, MD). Additionally, the human breast carcinoma lines, MCF-7 (HTB-22) and T-47D (HTB-133), a cervical carcinoma line, HeLa (CCL-2), an adrenocortical carcinoma line, SW-13 (CCL-105), a colon carcinoma cell line, Colo 201 (CCL-224), and a melanoma line, SK-MEL-3 (HTB-69), were also obtained from American Type Culture Collection. Cell lines were maintained in Dulbecco's Modified Eagles Media (DMEM) (Gibco, Grand Island, N.Y.) supplemented with 10% heat inactivated fetal bovine serum (Sigma, St. Louis Mo), 50 U/ml penicillin (Gibco), 50 µg/ml streptomycin (Gibco), 2 mM L-glutamine (Gibco), 0.1 mM Non Essential Amino Acids (Gibco), and 10 mM HEPES pH 7.2 (Gibco). In all experiments, cells were harvested in exponential phase growth.

The BRW cell line was established from a surgical specimen from a 26 year old male with an untreated primitive neuroectodermal tumor. It was serially passaged as a xenograft in Balb/C nu/nu female mice and was adapted to growth *in vitro* using the above conditions after its 22nd *in vivo* passage. It reacts strongly with anti-vimentin and does not react with anti-Glial-Fibrillary Acidic Protein using immunohistochemical staining.

Cytotoxicity experiments

Adherent cells were trypsinized, and placed into 96 well flat bottom tissue culture plates in 50 µl of supplemented DMEM at a concentration of 5×10^3 cells/well for 48 hour experiments and 2.5×10^3 cells/well for 8 day experiments. The cells were incubated overnight at 37° C. Gossypol was dissolved in Dimethyl Sulfoxide (DMSO) (Sigma), diluted to the desired concentration in DMEM and 50 µl/well was added to cells in sextuplet for each experimental condition. The vehicle was added to the six control wells.

Cell viability assay (MTT)

The Microculture Tetrazolium Assay was performed as previously described [22]. Briefly, when tetrazolium dye is added to viable cells, the reagent is metabolized by the mitochondria to form a purple formazan compound, and the optical density is proportional to the number of viable cells. After completion of the drug exposure, 20 µl of MTT (3-[4,5-Dimethyl-thiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) (Sigma) (5 mg/ml in phosphate buffered saline) was added to each well and incubated for 4 hours at 37° C. After incubation, 100 µl of lysing buffer was added to each well and incubated overnight at 37° C to solubilize the formazan. Lysing buffer was prepared from 20% w/v Sodium Dodecyl Sulfate (Sigma), which was dissolved in 50% N,N-dimethyl formamide (Sigma) and distilled water. Plates were read on a computerized microplate ELISA reader at an optical density of 570 nm. The percent cytotoxicity was determined by subtracting the ratio of treated optical densities to control optical densities (OD) from one and multiplying by 100. The concentration giving 50% cytotoxicity, IC50, was determined from the dose response curves for each cell line using the Sigma Plot software package. Each experiment was performed five separate

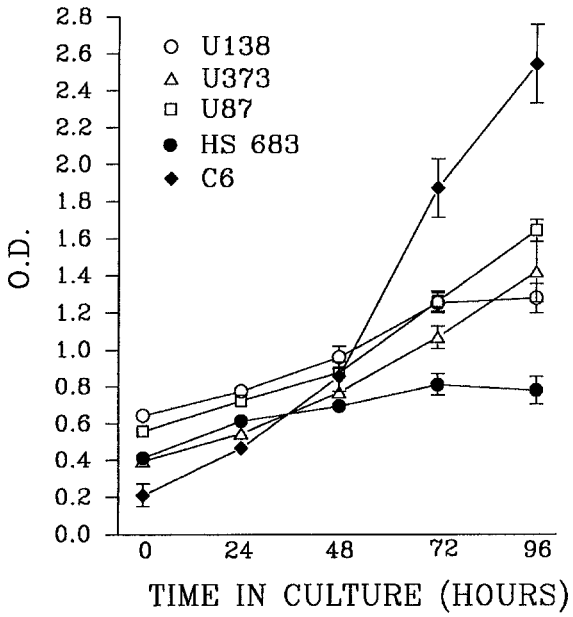

Fig. 2. The growth characteristics of five glioma cell lines as measured by the MTT assay. The rat glioma cell line, C6, was the fastest growing line, HS 683 was the slowest growing line and U373, U87 and U138 were intermediate. Each data point was calculated from sextuplet wells. The bars represent standard deviation.

times and the data presented are the pooled results of the five experiments.

Additionally, the cytotoxicity of gossypol to HS 683 was also measured using direct cell counting. In this experiment, cells were seeded into 24 well tissue culture plates at a concentration of 5×10^4 cells/well in a final volume of 1.5 ml supplemented DMEM and gossypol was added after a 24 hour incubation allowing for cell attachment. Gossypol was again dissolved in DMSO and added to the media. The concentrations of gossypol ranged from 0.1 μM to 100 μM. After 48 hours exposure, cells were trypsinized (0.5% Trypsin and 0.53 mM EDTA-4Na, Gibco), stained with trypan blue and counted in a hemocytometer. The data was pooled from 16 wells using 4 separate experiments.

LDH isoenzyme analysis

The LDH isoenzyme profile was performed by 1% agarose gel electrophoresis on 5 μl of sonicated cell lysate harvested from a single cell suspension of 1×10^7 cells essentially as described by Tuszynski [12]. Gels were then subjected to laser densitometry using a Barneyscan densitometer and integrator (Barneyscan Corp, Berkeley, CA) and the percentage of each isoenzyme was estimated as described by Benz [26]. The percentage of cathodal isoenzyme activity, type M, was calculated as follows: % M = % LDH5 + (0.75 × % LDH4) + (0.5 × % LDH3) + (0.25 × % LDH2). The LDH isoenzymes analysis was done on passages of each cell line concurrent with the cytotoxicity experiments.

Statistical analysis

All *in vitro* experimental conditions were performed in sextuplet in each experiment, and each experiment was repeated five times. Individual well optical densities served as the fundamental data and were subjected to a 3 or 4 way ANOVA. The significance of the differences of the IC50s for the cell lines was tested using Tukey's t Test to compare the IC50s for each of the five individual determinations of the IC50 for each line. The relationship of the LDH profile to the IC50 was tested by calculation of a linear correlation coefficient and the significance of the correlation was tested using the two tailed non directional Student's t Test.

Xenograft experiments

Balb/C nu/nu mice were fed and housed using sterile conditions as previously described [14, 25]. Mice were subcutaneously implanted with 5 × 5 mm fragments of tumor excised from the 26th *in vivo* passage of the BRW cell line and the tumor was allowed to grow for two weeks prior to gossypol treatment to enable the establishment of the xenograft. Eight xenografted animals were used as untreated controls and eight were treated with gossypol 30 mg/kg via gavage five days a week for four weeks. 0.2 M gossypol dissolved in DMSO was then diluted 1 : 50 in normal saline for orogastric administration. The animals were weighed weekly. Two weeks after the completion of gossypol therapy, the animals were sacrificed and the tumors excised and

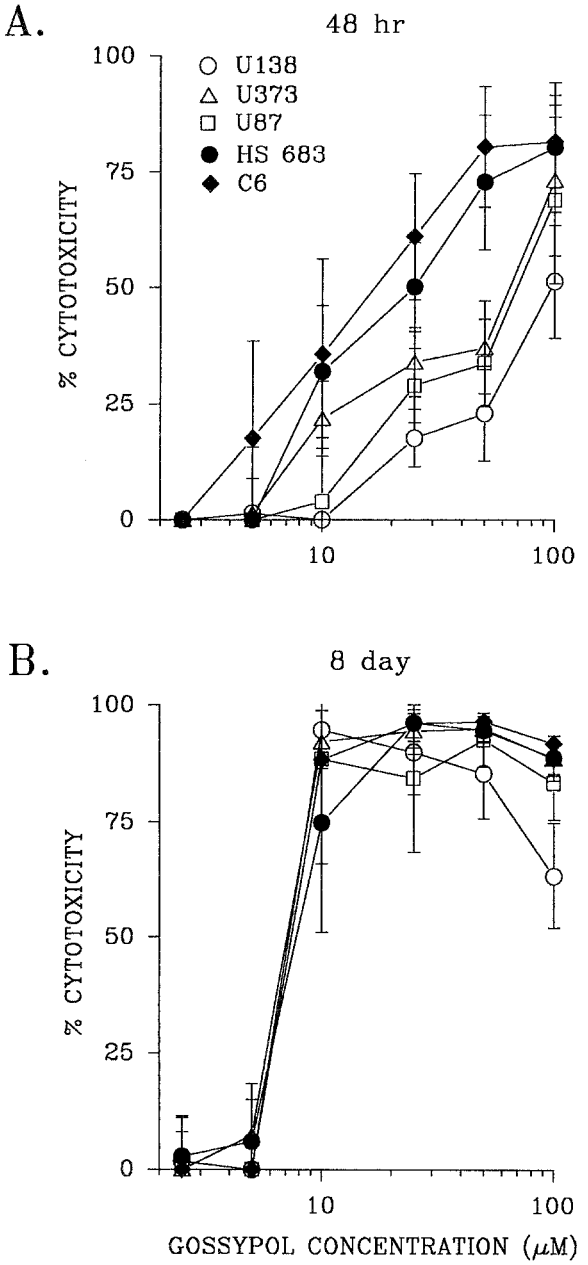

Fig. 3. The dose response curves as measured by the MTT assay for gossypol against C6, U373, HS 683, U87, and U138 at 48 hour (Fig. 3a) and 8 day (Fig. 3b) incubations. The % cytotoxicity is calculated as the (1-OD of treated wells/control wells) × 100.

weighed. The mean percent tumor growth inhibition was calculated as follows % TGI = 100 (1-mean weight of the treated animals/mean weight of control animals) [25].

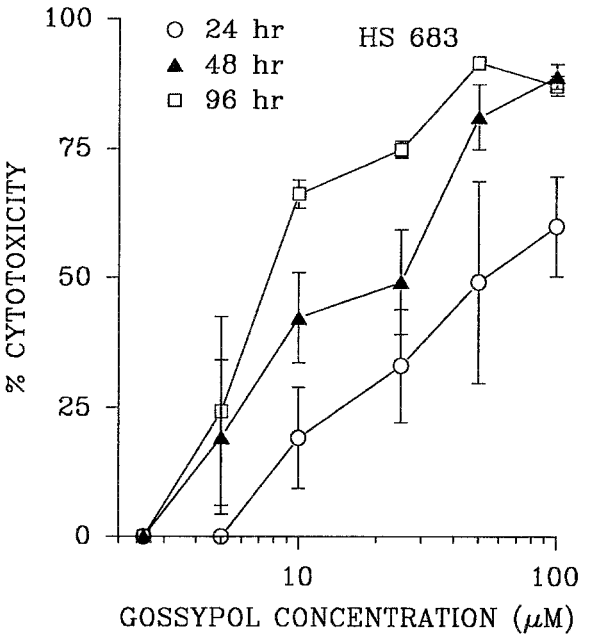

Fig. 4. The effect of increasing length of exposure on the cytotoxicity of gossypol. HS 683 was incubated with gossypol at concentrations ranging between 2.5 and 100 μM for 24, 48 and 96 hours prior to performing the MTT assay.

Results

The relative growth rates of the five glioma lines as measured by the MTT assay was assessed and the results are shown in Fig. 2. The rat glioma cell line, C6 was the fastest growing line, HS 683 was the slowest growing line and U138, U373, and U87 were intermediate.

Dose response curves to gossypol were then generated for the five cell lines using concentrations ranging between 2.5 μM and 100 μM for 48 hour and 8 day incubations. These results are shown in Fig. 3. A lower concentration of cells was plated for 8 days because the control wells became confluent at the higher plating concentration. The IC50 at 48 hours (concentration giving 50% cytotoxicity) was lowest for C6 at 18 μM, followed by HS 683 at 25 μM. U 373, U87 and U138 had higher IC50s at 64, 69 and 97 μM respectively. The IC50s for C6 and HS 683 were significantly lower when compared to the other three lines, but the difference between C6 and HS 683 was not statistically significant. There was no difference between U373 and U87, but both were statistically significantly lower than U138. The

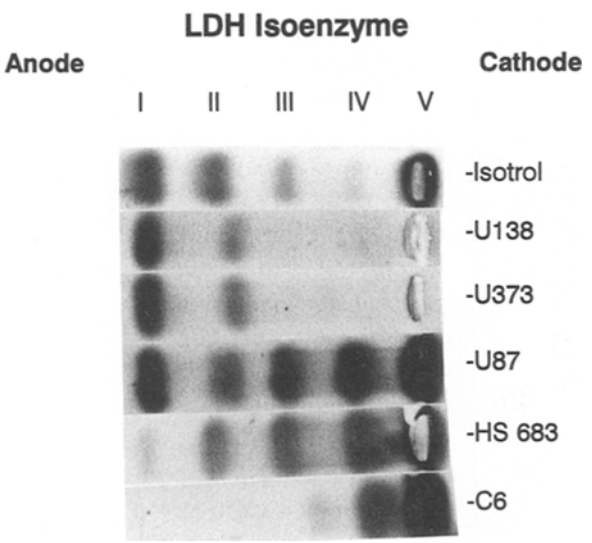

Fig. 5. The LDH isoenzyme pattern of the five glioma lines as determined by gel electrophoresis. The isoenzymes are numbered I (anodal)-V (cathodal). Isotrol is a control lysate containing all five isoenzymes. Each gel had been run with blank lanes surrounding each cell line lysate to allow for laser densitometry. The individual lanes were photographed and a composite made for ease of presentation.

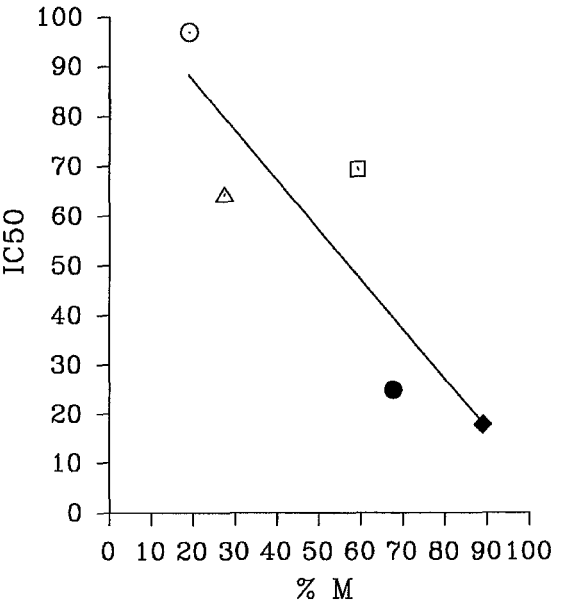

Fig. 6. The correlation of the IC50 of gossypol against 5 glioma cell lines with the percent of LDH subunit M. O-U138; Δ-U373; ❑-U87; ●-HS 683; ♦-C6.

p value for the statistically significant differences was $p < 0.01$ for all comparisons, except HS 683 vs. U373 and U138 vs. U87 where $p < 0.05$. The concentration of DMSO in wells treated with the highest concentration of gossypol was 0.1%. This concentration of DMSO had no cytotoxic effect on any of the cell lines when compared to untreated controls (data not shown).

When measured after 8 day incubations, the IC50s were lower for all tested cell lines, and the decrease was more marked for the more resistant lines. Additionally, there was no statistically significant difference in the IC50s between any of the cell lines when tested after 8 day incubations.

Cytotoxicity to gossypol was found to be both time and concentration dependent. Figure 4 shows the cytotoxicity of gossypol on HS 683 at 24, 48 and 96 hour incubations, demonstrating increased toxicity with increased length of incubation, as well as increased toxicity with increasing concentrations.

The sensivitity to gossypol of the five cell lines at 48 hour incubations was correlated to their LDH profiles as determined by gel electrophoresis and quantified by laser densitometry. Specifically, the cytotoxicity was related to the expression of LDH 5 and the percentage of the M subunit of LDH. The LDH profiles are shown in Fig. 5 and their quantification is summarized in Table 1. The linear regression line showing the correlation of the IC50s to the % M is shown in Fig. 6. The correlation coefficient was – 0.88 and the relationship was shown to be significant by Student's two tailed non directional T test with an alpha level of significance of 0.05.

We compared the cytotoxicity of gossypol to the glioma lines to its cytotoxicity to non central nervous system solid tumor cell lines at 48 hour and 8

Table 1. The distribution of the LDH isoenzymes and the percentage of LDH type M for each of the five glioma cell lines as quantified by laser densitometry

Cell line	I	II	III	IV	V	%M
U138	60	24	2	8	6	19
U373	53	24	1	4	18	28
U87	18	12	16	23	31	59
HS 683	1	13	26	34	26	68
C6	0	0	0	44	56	89

day incubations. The IC50s for all tested cell lines are summarized in Table 2. Our results on gossypol's activity against non-central nervous system solid tumors are comparable to those previously reported [12–15]. Additionally, we studied the effect of gossypol against BRW, a human CNS tumor cell line established in our laboratory from a patient with a primitive neuroectodermal tumor which had been established by serial passage as a nude mouse xenograft and adapted to growth in tissue culture after its 22nd passage. The LDH profile of BRW showed predominately LDH 4 and 5 with 29% LDH 5 and 63% M. The two more sensitive lines from our initial group, C6 and HS 683, as well as BRW had similar sensitivity to gossypol as the other solid tumor cell lines, some of which were selected because of their previous known sensitivity to gossypol. U138, U373 and U87 were less sensitive than the non central nervous system solid tumor lines.

Because gossypol is an inhibitor of mitochondrial energy metabolism, it is conceivable that the cytotoxicity of gossypol would be overestimated by the MTT assay. In order to test this hypothesis we determined the IC50 of gossypol on HS 683 at 48 hours using cell counting with trypan blue as the end point rather than MTT, and these data are shown in Fig. 7. Interestingly, the IC50 was 4 μM lower than the 25 μM determined by the MTT method.

Finally, we undertook an experiment to determine the *in vivo* activity of gossypol against BRW in the nude mouse xenograft model. The BRW xenograft was harvested from an untreated mouse, then unilaterally subcutaneously implanted into Balb/C nu/nu mice, and two weeks were allowed for the establishment of the xenograft prior to treatment. The treated group of eight mice was given gossypol 30 mg/kg via gavage five times a week for four weeks and these were compared to 8 untreated controls. There was one xenograft in the control group which failed to take and did not grow at all during the time course of the experiment and this animal was eliminated from analysis. Additionally eight xenografted animals were treated with the vehicle but without gossypol by gavage, and no significant difference in tumor or animal weight was seen when compared to untreated controls. Two weeks after the completion of therapy, the animals were sacrificed and the tumors excised and weighed. The mean tumor weights were 2.5 grams in the gossypol treated group and 5.2 grams in the untreated control group showing a statistical difference at $p < 0.01$ by Tukey's t Test. The mean tumor growth inhibition was 52%. Regression of the tumors was not seen. The animals were weighed weekly. The treat-

Table 2. The IC50 of gossypol at 48 hour and 8 day incubations against CNS and non CNS solid tumor cell lines

Cell line	48 hr	8 day
MCF-7	10	5
Colo 201	15	4
HeLa	18	4
C6	18	9
BRW	20	7
SW-13	20	6
T-47D	20	5
HS 683	25	9
SK-MEL-3	42	4
U373	64	7
U87	69	8
U138	97	9

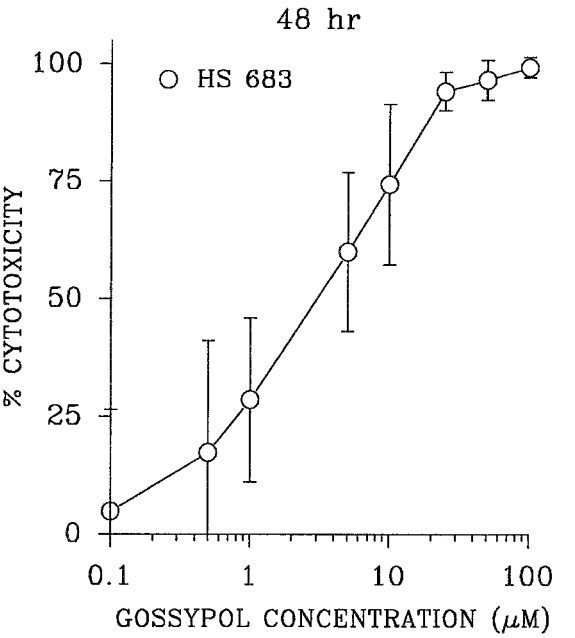

Fig. 7. The cytotoxicity of gossypol at 48 hours against HS 683 as determined by direct cell counting. The % cytotoxicity was measured as the 1-(# cells treated group/# cells control group) × 100.

ed group appeared well and there were no treatment related fatalities. The treated group did not lose weight, but the control group gained statistically significantly more weight. However the differences in the weight gain 6 weeks after the initiation of gossypol treatment, 2.7 grams, approximated the difference in the mean tumor weights between the two groups.

Discussion

The antiproliferative effects of gossypol on one rat and five human glioma cell lines was demonstrated using the MTT assay. At 48 hour incubations, these effects were more pronounced in glioma cell lines that contained greater percentages of LDH isoenzyme 5. The *in vitro* antiproliferative effects of gossypol are concentration and time dependent. More prolonged incubation times (8 days) markedly decreased the IC50s of the tested lines, particularly the more resistant ones, and there was no significant difference in the sensitivity between the various lines with this more potent incubation. Our results confirm the observation that longer exposure times to gossypol increase the cytotoxicity, as compared to well known behavior of conventional agents, such as BCNU, which in our laboratory has only minor increases in cytotoxicity with more prolonged incubations. As opposed to Rhodamine-123, another anti-mitochondrial agent with rapid onset of action, shorter exposure times to gossypol have been previously shown to have a relatively less pronounced cytotoxicity, and the IC50 has been shown to continue to decline with incubation times of up to four days, then plateau [15]. The mechanism(s) of this effect is unclear but may include instability of the drug, time dependence of its antimitochondrial effects, time dependence of its effects on membranes, or a complex interaction of multiple cellular effects. It would appear that MTT algorithms designed for conventionally acting cytotoxic agents, where the drug is washed from the media after a short incubation time [24], may not be appropriate to study the antiproliferative effects of gossypol.

The cytotoxicity of gossypol on the three more sensitive central nervous system tumor cell lines were comparable to its effect on the other non-central nervous system solid tumor lines tested, several of which had been previously reported to be sensitive to gossypol. We tested these other lines to directly compare gossypol's cytotoxicity, because the absolute values of the observed IC50s are likely to be method dependent as well as cell line dependent. The previous reports on gossypol's activity in other solid tumors have used a variety of assays to demonstrate the cytotoxicity. It was conceivable that gossypol's action would be overestimated by the MTT assay because of its antimitochondrial effect and the dependence of the MTT method on the mitochondrial reduction of the tetrazolium dye. Surprisingly, the concurrent cytotoxicity experiment on HS 683 using direct cell counting as an endpoint showed a lower IC50 then was demonstrated with the MTT method. Another instance where the MTT underestimated the cytotoxicity as compared to cell counting has been reported recently in the testing of the cytotoxicity of an agent called Genestein against MCF-7 breast cancer, Jurkat T cell leukemia and L-929 transformed fibroblasts cells [27]. Those authors demonstrated that Genestein had caused an accumulation of mitochondria in cells which were blocked in G2/M phase of the cell cycle. The mechanism of the disparity with gossypol is not immediately apparent and is under further study.

The cytotoxic effect of gossypol is most likely through its inhibition of energy metabolism. It accumulates in mitochondria and it has been shown to cause selective destruction of these organelles at 10 μM concentrations in other solid tumor cell lines *in vitro* [28]. Gossypol is a Schiff base forming agent, a chelator of metal ions and an inhibitor of multiple enzymes [29], particularly enzymes which involve redox reactions and employ NAD or NAD (P) cofactors. In spermatozoa, it is controversial whether the mechanism of gossypol's inhibition is through the inhibition of glycolysis [17, 18], and/or oxidative phosphorylation and electron transport [16]. Of interest, it has been previously shown in several solid tumor cell lines that sensitivity to gossypol *in vitro* correlated to LDH isoenzyme profiles [12, 13]. Our data confirm this observation in glioma cell lines. We were not able to demonstrate any sta-

tistical correlation at 8 days incubation, perhaps because the more potent cytotoxicity at these long incubations overwhelms any subtler differences in sensitivity between lines. Additionally, the lower target cell to drug ratio that was used in our 8 day incubation experiments may have also increased the cytotoxicity [15]. If gossypol were shown to have any activity in clinical trials, the correlation between the LDH profile of the resected tumor specimens and response to gossypol could be determined as a corollary to these trials. If such a correlation were demonstrated, the LDH profile could then be tested for its ability to predict the clinical response to gossypol treatment.

Gossypol has been tested *in vivo* against the Ehrlich ascites tumor cell line implanted into NMRI mice and inhibited tumor growth in a dose dependent fashion [30]. At higher doses, weight loss and hepatotoxic death of the mice were seen. Rao *et al.* [31] tested gossypol given as a single intraperitoneal injection in BDF1 mice bearing the L1210 or P388 leukemia lines or the CA 755 mammary carcinoma line. Little activity was seen against the rapidly growing leukemia lines but 66% of the mice with slower growing CA 755 were rendered tumor free at the optimal dose of 0.5 mg/mouse, again with toxicity at higher doses. Wu *et al.* [14] tested gossypol, given at a dose of 30 mg/kg daily via orogastric tube in nude mice implanted with the SW-13 adrenocortical carcinoma line. Treated mice had a 54% tumor prevalence at 5 weeks as compared to 95% in controls.

Although tumor regressions were not observed in our *in vivo* study of gossypol on the BRW primitive neuroectodermal tumor xenograft, treatment with gossypol did have a significant effect on delay of tumor growth as defined by a % TGI of greater than 50%. The route and schedule used was similar to that described by Wu *et al.* and our results confirm that oral gossypol treatment was well tolerated by the mice. Collectively, these preliminary *in vivo* studies [14, 30, 31] provide further evidence of the cytotoxic effect of gossypol seen in previous tissue culture studies, and our current work extends these observations to primary central nervous system tumors.

Gossypol has been tested extensively in humans by Chinese investigators as a male contraceptive, as it had been found to cause azoospermia and inhibit sperm motility in greater than 99% of patients tested and these effects were reversible in 90% [19]. At relatively high doses in humans (60–70 mg/day orally for 35–42 days), side effects included necrospermia, anorexia, fatigue, dry mouth, elevated transaminases, diarrhea, and upper abdominal discomfort. At lower dose schedules, such as 20 mg/day for 6 months, treatment was better tolerated and side effects consisted of transient weakness (13%), increased appetite (3%), decreased appetite (2%), epigastric distress (20%), and nausea (1%). Hypokalemia was seen in 10% of Chinese patients and was thought to be the dose limiting toxicity. Interestingly, hypokalemia was not found to be a problem in clinical trials conducted outside of China [32].

There have been two published clinical studies of gossypol as an antineoplastic agent. A phase I study done by Stein *et al.* [20] in a group of patients with variety of solid tumors, not including primary brain tumors, found that the maximal tolerated dose was 30 mg daily or 120 mg one time a week with the dose limiting toxicity's being emesis. The treatment was otherwise very well tolerated. No clinical efficacy was seen in the 20 evaluable patients, but the study was not designed to evaluate the response rate. Flack *et al.* [21] have performed a Phase II trial of gossypol in the treatment of metastatic adrenocortical carcinoma, a disease generally considered resistant to conventional chemotherapy. They treated 21 patients with gossypol in doses ranging between 30 mg/day and 70 mg/day orally. Three patients rapidly died of disease and were not considered assessable. There were three partial responses, one patient with a minor response, one patient with stable disease, and thirteen patients with progressive disease. Toxicity included xerostomia in 93%, dry skin in 71%, fatigue in 64%, nausea in 36%, vomiting in 21%, alopecia in 14% and gynecomastia in 7%. The most serious side effect was transient ileus, which was seen in 21%. No myelosuppression was seen in either trial.

Gossypol is highly lipid soluble at physiological pH, and, in humans, has a long serum half life of 3 weeks [33]. Both features make this drug attractive

for potential use against glioma. The lack of myelosuppression and low level of serious toxic effects suggest that gossypol may be a drug which older patients could tolerate. Furthermore, if single agent activity is demonstrated, gossypol may be attractive for use in combination chemotherapy regimens.

Lonidamine is another antimitochondrial agent which inhibits lactate production by specifically inhibiting mitochondrially bound hexokinase. Lonidamine has been used in a phase II trial in recurrent malignant glioma in an attempt to exploit a metabolic strategy, and 2 partial responses in 10 assessable patients were seen [34]. Further trials with lonidamine are in progress [35].

In this study we have shown that gossypol has *in vitro* anti-glioma activity in human central nervous system tumor cell lines and the activity correlated to the LDH isoenzyme profile. Additionally, we have demonstrated a significant effect on tumor growth delay *in vivo* using the nude mouse xenograft model in one of these lines. Since gossypol has been well tolerated in previous contraceptive and antineoplastic clinical trials, we believe that further preclinical and clinical studies of gossypol in the treatment of gliomas should be pursued.

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