Expression of Anti-apoptosis Genes Determines the Response of Adrenal Cancer to Apoptosis-inducing Chemotherapy

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Abstract. *Background: This study tested the hypothesis that response of adrenal cortical carcinoma (ACC) to pro-apoptosis drugs depends on expression of anti-apoptosis genes. Materials and Methods: Expression of Bcl-2 and Bcl-XL proteins was determined in two human adrenal cancer cell lines, NCI-H-295 and RL-251. Two pro-apoptosis drugs, gossypol (G) and docetaxel (D) were tested in vitro and in vivo in a human ACC/SCID mouse chimera. Results: Bcl-XL was strongly expressed in RL-251 but not in H-295 and neither expressed the Bcl-2 protein. G and D induced greater dose-dependent inhibition of cell proliferation in RL-251 than in H-295 cells and completely suppressed growth of tumors with high expression of Bcl-XL ($p<0.05$) while there was no growth suppression in tumors without Bcl-XL expression. Conclusion: This study provided proof of concept that expression of Bcl-XL determines response to pro-apoptosis drugs. Profiling adrenal tumors for expression of anti-apoptosis genes may provide clues to their potential response to drugs that induce apoptosis.*

Adrenal cortical carcinomas (ACCs) are rare but highly malignant tumors with poor prognosis. Treatment of patients with adrenal cancer includes surgery, systemic chemotherapy and mitotane. Surgical resection is effective in early stage disease but not curative for most patients because of occult micrometastases. In advanced ACC, chemotherapy gives variable results (1) and better molecular targeted therapies are needed (2). The ability to induce apoptosis is an important feature of anticancer drugs. Factors that function as antagonists of apoptosis in human cancers include the Bcl-2 and Bcl-XL proteins. The Bcl-XL protein appears the most important. When overexpressed, Bcl-2 and Bcl-XL confer resistance to chemotherapeutic agents or radiation therapy (3, 4). Bax, another protein involved in the apoptosis cascade, has pro-apoptosis function; a low expression of Bax makes tumors resistant to treatment (5). (–)-Gossypol, a lipid soluble, polyphenolic compound isolated from cottonseed oil has anti-proliferative activity *in vitro* against a variety of human solid tumor cell lines and has been tested *in vivo* in patients with ACC with modest response (6). (–)-Gossypol is a potent inhibitor of Bcl-XL and a moderately potent inhibitor of Bcl-2. This suggests that the anti-tumor activity of gossypol is due partly to inhibition of the anti-apoptotic activity of Bcl-XL and that it is able to enhance sensitivity of tumor cells with high Bcl-XL expression to other chemotherapeutic agents (7). Docetaxel, a semi-synthetic agent from the taxoid family, is an effective drug for chemotherapeutically resistant breast and non-small cell lung cancer. Its mechanism of action also involves promotion of apoptosis (8).

The present study tested the hypothesis that adrenal tumors that express anti-apoptosis genes are most responsive to treatment with pro-apoptosis drugs. It was also hypothesized that (–)-gossypol and docetaxel singly or in combination will induce apoptosis in cancer cells with high expression of Bcl-XL, but will have little effect on cancer with low Bcl-XL and Bcl-2. This study examined two human ACC cell lines with different expression of anti-apoptosis genes: a steroid secreting cell line, NCI-H-295, with low expression and a CXC chemokine-producing cell line with high expression of Bcl-X, RL-251.

Materials and Methods

Two cell lines were used: RL-251 was established in the authors' laboratory from a poorly differentiated ACC with abundant inflammatory cells (9). It produces several CXC-chemokines, including IL-8, ENA-78, Gro α, Gro γ and NAP-2. NCI-H-295 was established by Gazdar *et al*. (10) and was obtained from the American Type Culture Collection (ATCC; Manassas,VA, USA). It

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produces multiple steroids, including cortisol, aldosterone and DHEA. (–)-Gossypol was prepared in Dr Wang's laboratory and docetaxel was provided by Aventis (Bridgewater, NJ, USA).

Characterization of Bcl-2, Bcl-XL and Bax proteins in RL-251 and H-295 cell lines. Expression of these proteins was detected by Western blot analysis. Logarithmically growing cells were harvested at 75% confluence. Protein extracts were resolved on SDS-PAGE under denaturing conditions and proteins immobilized on PVDF membranes. The membranes were probed with primary antibodies (mouse anti-Bcl-XL monoclonal antibody H-5 at 2 mg/ml; mouse anti-Bax monoclonal antibody YTH-2D2 at 4 mg/ml; mouse anti-Bcl-2 monoclonal antibody YTH-8C8 at 2 mg/ml or GAPDH at 1:100,000 dilution and sheep anti-mouse as secondary antibody) and the blots developed using the ECL system. All experiments were performed in duplicate and protein expression was graded as negative (no band observed), weakly positive, moderately positive or strongly positive relative to the intensity of the GAPDH bands. There was strong expression of Bcl-XL in RL-251 cells but negligible or no expression in H-295 cells. The cell line RL-251 also showed a band of strong intensity corresponding to Bax while H-295 showed only a signal of weak intensity. Neither of the two cell lines showed expression of Bcl-2 protein (Figure 1).

In vitro studies. Cells were incubated in Gibco RPMI 1640 media, supplemented with HITES (hydrocortisone, insulin, transferrin [Sigma], estradiol, and sodium selenite), and 5% fetal bovine serum and plated in 96-well microtiter plates in 100 μl at plating densities of 2,000 cells/well for RL-251, and 10,000 cells/well for H-295. The plates were incubated at 37˚C, 5% CO_2, 95% air and 100% relative humidity for a 48-hour period. One plate of each cell line was fixed with 16% trichloroacetic acid (TCA), to represent a measurement of the cell population at the time of drug addition. Drugs were added at four different concentrations. All experiments were done in triplicate. Cell doubling was measured by sulforhodamine B assay (SRB) at day five for RL-251 and day seven for H-295. An IC_{50} dose was determined.

In vivo studies. (–)-Gossypol and docetaxel were tested *in vivo* in a human adrenocortical carcinoma/SCID mouse chimera using the two cell lines described above. Cells were injected into the subcutaneous tissue of each flank. Ten mice were used for each cell line and treatment group. Tumors became palpable after 15 days in RL-251 injected animals and 30-45 days in animals injected with H-295 cells. Drug treatment was initiated when tumors became palpable. Docetaxel was injected intravenously into the tail vein in a single weekly dose of 10 mg/kg for 3 weeks and (–)-gossypol was given orally in a dose of 15 mg/kg daily for 4 weeks. Tumor size was measured every 3 days with calipers and the volume of the xenograft calculated as $a \times b^2/2$, where a is the longer and b, the shorter axis of the tumor. At the end of the study, animals were euthanized, tumors removed and weighed in a Mettler balance, and blood collected from the eye socket, following enucleation. Body, spleen and liver weights and polymorphonuclear (PMN) cell counts were recorded. The animals were observed for changes in food intake, quality of their coat and degree of physical activity. The measurement of spleen and liver weight and PMN counts were performed because mice with RL-251 xenografts experience marked splenomegaly, neutrophilia and PMN infiltration of the livers on account of their production of CXC chemokines with angiogenic and chemotactic activity. These measurements were expected to be useful biomarkers of drug effects. The protocol was approved by the University Committee for the Use and Care of Animals (UCUCA). Data from the *in vitro* and *in vivo* experiments were collected for the two cell lines and the three treatment conditions, ((–)-gossypol, docetaxel and combination of the two drugs) and analyzed by analysis of variance (ANOVA). The predicted growth curves per treatment category over time of the two types of tumors in response to various treatments were compared statistically. For this comparison, a mixed-effects model prediction was used, where the left- and right-sided tumors were clustered within each animal, and data from the animals were allowed to have random deviation in intercept (treatment initiation and slope growth over time) around a group mean for the treatment received.

Results

In vitro studies. The addition of increasing concentrations of (–)-gossypol and docetaxel to the two cell lines, showed that the RL-251 cells with expression of Bcl-XL were much more sensitive to suppression of cell proliferation than the H-295 cells without this expression. This difference in response is depicted by differences in IC_{50} dose, showing a statistically significant ($p<0.01$), six-fold greater sensitivity to (–)-gossypol and a ten-fold greater sensitivity to docetaxel for the cells with expression of Bcl-XL (Table I).

In vivo studies. Mice with RL-251 tumors that express Bcl-XL showed marked suppression of tumor growth with (–)-gossypol alone, docetaxel alone or their combination (Figure 2A). In contrast, no such effect was observed in mice with the H-295 xenografts (Figure 2B). In addition to the suppression of tumor growth in the RL-251 animals, there was marked suppression of splenic enlargement and of the leukocytosis and neutrophilia observed in the untreated animals. The drugs were well tolerated by the animals and there was no significant change in food intake, body weight or kidney weight with treatment. The differences in tumor growth rate are also depicted by calculating the predicted tumor weight by treatment categories (Figure 3). When compared to control, the addition of (–)-gossypol, docetaxel or their combination caused statistically significant ($p<0.05$) greater suppression of tumor growth in the RL-251 cells with expression of Bcl-XL than in the H-295 cells without such expression.

Discussion

The present study tested the hypothesis that adrenal tumors that express anti-apoptosis genes are most responsive to treatment with pro-apoptosis drugs. It was also hypothesized that (–)-gossypol and docetaxel singly or in combination will induce apoptosis in cancer cells with high expression of Bcl-XL, but will have little effect on cancer with low Bcl-XL. Two human ACC cell lines were studied with different expression of anti-apoptosis genes: a steroid secreting cell line, NCI-H-

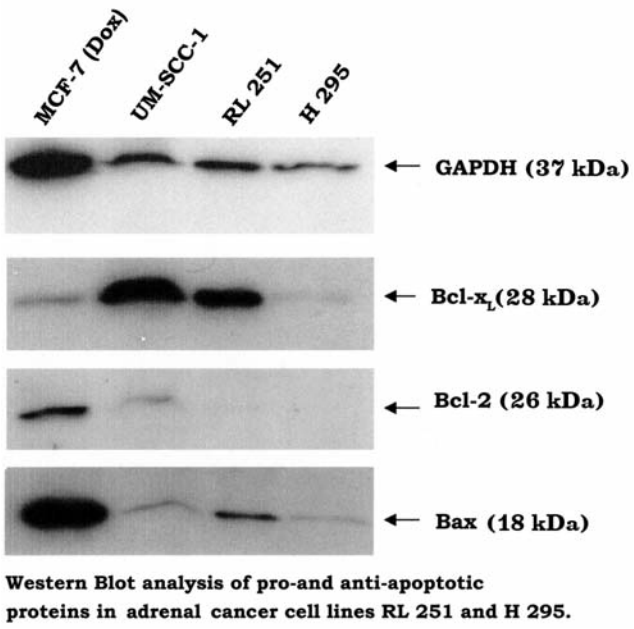

Figure 1. *Western blot analysis of pro- and anti-apoptosis proteins in adrenal cancer cell lines RL-251 and NCI-H-295. There were marked differences in Bcl-XL and BAX between the cell lines.*

295, with low expression and a CXC chemokine-producing cell line with high expression of Bcl-XL, RL-251. It was found that gossypol alone, docetaxel alone and their combination completely suppressed growth of tumors with high expression of Bcl-XL ($p<0.05$) while there was no significant change in growth of tumors without Bcl-XL expression, providing proof of concept to the study hypothesis. These results provide a rationale for pre-selecting patients with ACC who may benefit from the tested types of therapy (gossypol and/or docetaxel) on the basis of the examined genetic profiles. It would have been ideal to study more than two cell lines of ACC in order to confirm these finding but only two well-characterized cell lines of human ACC are currently available. ACC is a rare but highly malignant type of cancer and patients have limited life expectancy when diagnosed in advanced stages. They have generally unsatisfactory response to systemic chemotherapy. In early stages (stages I and II) or in advanced stages with few isolated metastases, surgical resection may induce remissions lasting several years (1). More effective treatment of patients with ACC is likely to result from better understanding of the molecular mechanisms involved in tumorigenesis and cancer progression (2). In fact, profiling of adrenal cortical tumors has led to targeted therapy focused on specific genetic defects (11). The present study provided support to the possibility that profiling for anti-apoptosis genes may help identify tumors with a specific susceptibility to pro-apoptosis drug therapy.

Table I. *IC_{50} response of the two cell lines, RL-251 and H-295, to the addition of (–)-gossypol and docetaxel in vitro.*

	IC_{50} response (nM concentration)	
	(–)-Gossypol	Docetaxel
RL-251 cells	80	0.3
H-295 cells	500	30

Difference in response between cell lines: $p<0.01$.

Earlier studies showed that gossypol has activity against human adrenal cancer xenografts in nude mice. A clinical study extended these observations by using natural gossypol in patients with advanced stage IV ACC (6). In a phase I/II open-label clinical trial, 21 patients received 30-70 mg racemic gossypol per day; 18 patients completed six weeks of treatment. Partial response lasting several months to one year was observed in three patients and one patient had stable disease; however, the majority of patients (77%) had disease progression. Interest in gossypol as therapy for adrenal cortical carcinoma was strengthened by more recent preclinical studies that provided robust rationale for the use of gossypol as an antitumor drug. These preclinical studies raised the possibility that expression of apoptosis-regulating mechanisms is able to determine the response of ACC to pro-apoptosis drugs (7).

Gossypol is a polyphenolic compound derived from cottonseeds that possesses anti-proliferative and pro-apoptotic effects in various types of cancer cells. Of the two enantiomers present in the natural racemic mixture, (–)-gossypol is the most active (12). It has been found that (–)-gossypol inhibits the function of Bcl-2 and Bcl-XL in human prostate tumors in nude mice xenografts, increasing apoptosis or programmed cell death and making the cancer more sensitive to radiation and chemotherapy (13).

When tested in two cell lines of metastatic rat prostate cancer with different invasive ability, (–)-gossypol was most active in the cell line with the highest expression of Bcl-2 and Bcl-XL (14). Similar effects were reported in human prostate cancer cells. (–)-gossypol treatment caused growth suppression of the DU-145 cells and this effect was influenced through cell cycle regulators; (–)-gossypol down-regulated cyclin-D1, Rb, CDK4 and CDK6 and up-regulated p21 and TGF-β1 at the mRNA and/or protein levels (15). The mediating effect of TGF-β1 was confirmed in human, androgen-independent PC3 cells. In this setting, the stimulatory effect of (–)-gossypol on TGF-β1 expression correlated with its inhibitory effect on PC3 cell DNA synthesis and its ability to arrest cells in G0/G1 phase (16). Further studies on this cell line indicated that (–)-gossypol acts as a chemosensitizer, synergistically enhancing the antitumor activity of docetaxel, also an apoptosis-inducing

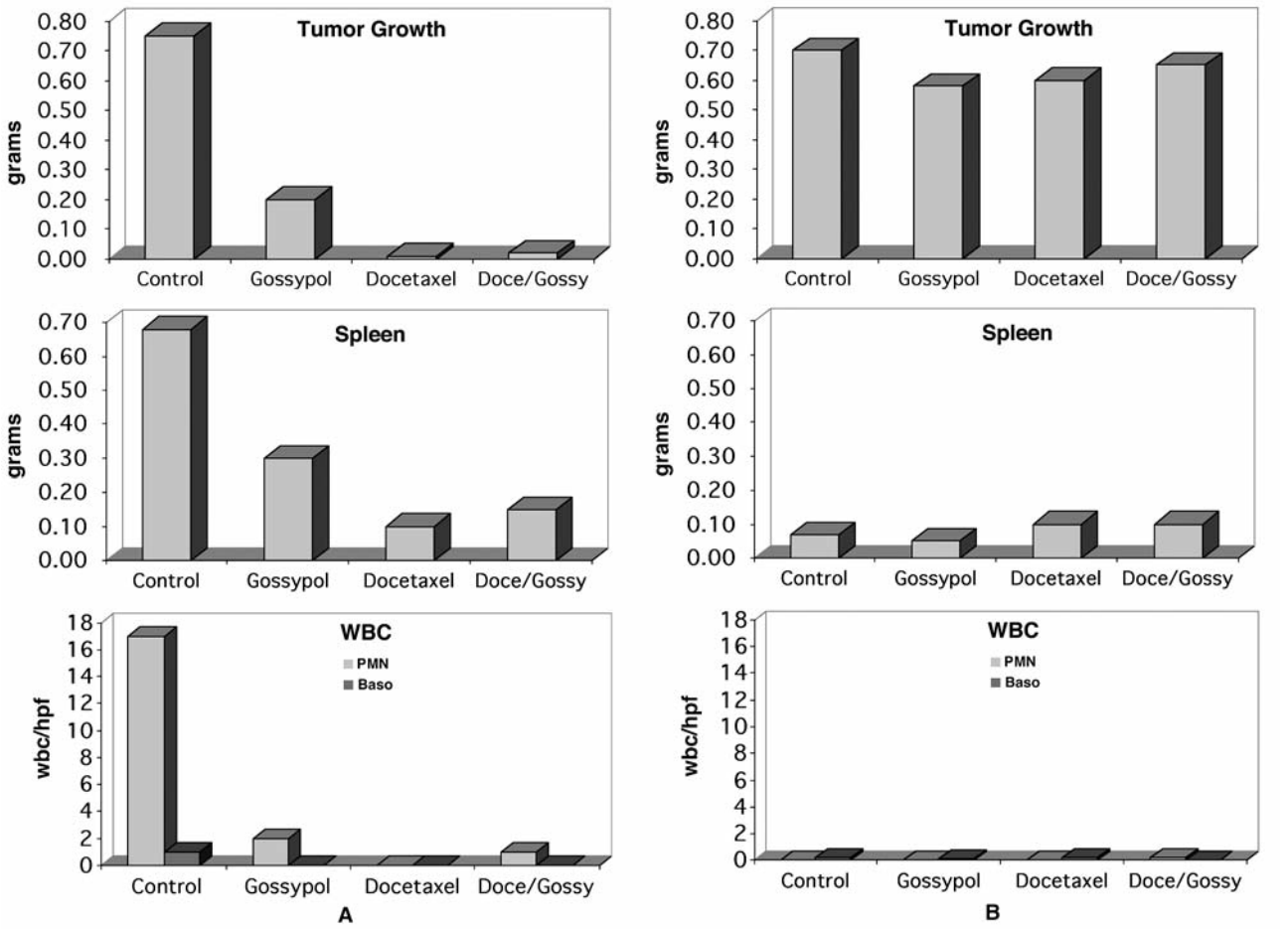

Figure 2. *In vivo response to (–)-gossypol, docetaxel and their combination (denoted as Doce/Gossy), with respect to tumour growth, spleen size, and white blood cell (WBC) count. A: Mice with RL-251 tumors with expression of Bcl-XL showed marked response to (–)-gossypol, docetaxel and their combination. There was also a reduction of spleen size and number of neutrophils observed in the control animals. B: Mice with H-295 tumors without expression of Bcl-XL showed lack of response to the tested drugs. Baso, Basophils; PMN: polymorphonuclear leukocytes.*

drug, both *in vitro* and *in vivo*. This effect appears to require an increase in pro-apoptosis proteins, PUMA and NOXA, which are well-known p53-inducible, pro-apoptotic members of the Bcl-2 family. In healthy cells, PUMA but not NOXA induces mitochondrial outer membrane permeabilization, and this function is mediated, in part, by a pathway that involves calcium release from the endoplasmic reticulum and the subsequent caspase activation. However, upon E1A oncoprotein expression, cells also become susceptible to outer membrane permeabilization induction by NOXA, owing their sensitization to the endoplasmic reticulum-independent pathway (17). The preclinical *in vitro* and *in vivo* studies described above, have led to phase II clinical trials of (–)-gossypol as adjunct therapy for prostate cancer.

Docetaxel is another antitumor drug that induces apoptosis. Initially described as an antimitotic agent, docetaxel binds to β-tubulins and stabilizes the microtubular network, leading to a block in the cell cycle at G_2-M and subsequent apoptosis of the cells (7).

The present study examined the possibility that (–)-gossypol is effective in suppressing ACC, especially in tumors with high expression of anti-apoptosis genes. If it were possible for the expression of these genes to be determined prior to treatment, those with high expression would be the ideal candidates for treatment with gossypol. Similarly, because of the chemosensitizing effect of (–)-gossypol, the study also tested the antitumor effect of combining gossypol with docetaxel, another apoptosis-inducing drug, in a synergistic combination in adrenal cancer. These studies did not investigate the mechanism of apoptosis in these ACC cell lines. This mechanism has been previously reported in prostate cancer (17) and the results of the present study were consistent with those in prostate cancer cells. In conclusion, the present study provided a

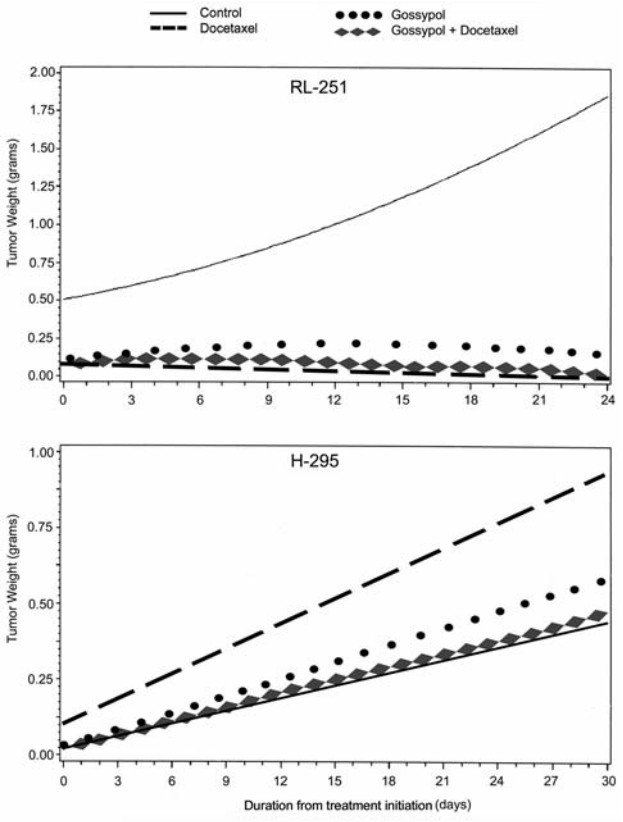

Figure 3. *Predicted growth curves per treatment category over time of the two types of tumors in response to treatment with (–)-gossypol, docetaxel or a combination of the two drugs. Only tumors with high expression of Bcl-XL responded to treatment.*

rationale for using (–)-gossypol alone or combined with docetaxel in phase I/II clinical trials in patients with adrenal cancer with high expression of anti-apoptosis genes.

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