Anti-prostate cancer activity of a ß-carboline alkaloid enriched extract from *Rauwolfia vomitoria*

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Abstract. The tropical shrub, *Rauwolfia vomitoria*, is a medicinal plant used traditionally to treat a variety of ailments. A bioactive ß-carboline alkaloid, alstonine, present in this extract was previously shown to have anti-cancer activity against cancer cell lines. This study considers the potential anti-prostate cancer activity of this extract *in vitro* and *in vivo*. *Rauwolfia vomitoria* extract standardized for ß-carboline alkaloids was tested for ability to influence the growth and survival of the human LNCaP prostate cancer cell line. A WST-1 assay was used to measure cell growth, and cell cycle analyses were conducted with flow cytometry. Western blot detection of PARP cleavage and accumulation of cells containing sub-genomic DNA indicated induction of apoptosis. Pathway specific microarray analyses were utilized to identify the effect of *Rauwolfia* extract on the expression of 225 genes. Mice xenografted with LNCaP cells were treated with the extract or placebo control, and tumor growth was measured for 5 weeks. The effects of the extract on xenografted tumor cell proliferation and apoptosis were measured by *in situ* BrdU incorporation and TUNEL staining. *Rauwolfia* extract decreased *in vitro* cell growth in a dose-dependent manner ($p<0.001$) and induced the accumulation of G1 phase cells. PARP cleavage demonstrated that apoptosis was induced only at the highest concentration tested (500 μg/ml) which was confirmed by detection of cells containing sub-genomic DNA. The expression of genes associated with DNA damage signaling pathway was up-regulated by *Rauwolfia* treatment, including that of GADD153 and MDG. The expression of a few cell cycle genes (p21, cyclin D1 and E2F1) was also modulated. These alterations were confirmed by RT-PCR. Tumor volumes were decreased by 60%, 70% and 58% in the groups fed the 75, 37.5 or 7.5 mg/kg *Rauwolfia*, respectively (Kruskal-Wallis test, $p<0.001$). The *Rauwolfia vomitoria* extract significantly suppressed the growth and cell cycle progression of LNCaP cells, *in vitro* and *in vivo*.

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Introduction

Prostate cancer is predicted to be the third leading cause of cancer-related deaths among men in the USA in 2006 (1). Traditionally, chemotherapy and radiotherapy have not proven to provide significant survival benefits to patients with advanced prostate cancer and most treatment options available are only palliative. Recent studies on taxane derivatives alone and in combination with other chemotherapeutic agents have demonstrated some limited benefit (2), but a need for more effective and less toxic means to target and/or prevent this disease clearly exists.

Natural products have long proven to be a bountiful resource for identification of bioactive compounds used in the treatment of a variety of ailments and diseases, including cancer. The taxane derivatives currently being used for the treatment of hormone-independent prostate cancer are but one example among many of the importance of this resource. However, systematic characterization of natural product and herbal therapies and identification of their mechanism(s) of action are crucial for the development of safe and efficacious therapies for prostate cancer prevention and treatment. Regarding this, we have begun to study a unique extract derived from the root bark of a plant found in the tropical secondary forests of Africa, *Rauwolfia vomitoria* (family: Apocynaceae) to determine whether it might have activity against prostate cancer. Various parts of this plant have been used as a traditional medicine for centuries to treat a variety of ailments including fever, general weakness, intestinal diseases, liver problems and mental disorders (3,4). Extracts from the root bark of this plant are enriched for compounds of the ß-carboline alkaloid family of which the main constituent is alstonine. This compound has been previously reported to reduce tumor cell growth in mice inoculated with YC8 lymphoma cells or Ehrlich ascitic cells (5). The data presented herein suggest that this plant extract has anti-prostate cancer activity in both *in vitro* and *in vivo* model systems which, based upon our analyses of gene expression patterns of treated prostate cancer cells, may be modulated by its effects on DNA damage and cell cycle control signaling pathways.

Materials and methods

Cell culture studies. The androgen-sensitive human prostate cancer cell line, LNCaP, was obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), L-glutamine (Gibco® Invitrogen Corp.). Erythromycin was included in the medium at a concentration of 100 mg/l (Sigma). The cells were maintained at 37˚C in a humidified atmosphere of 95% air and 5% CO_2.

Rauwolfia extract preparation. *Rauwolfia vomitoria* root bark extract was supplied in powdered form by Natural Source International, Ltd. (New York, NY). A single batch of extract was used for all studies here, and the composition of this extract was determined by HPLC to be 57% (w/w) ß-carboline alkaloids, with reserpine removed. For testing in *in vitro* and *in vivo* studies, the extract was completely dissolved in deionized distilled water and filtered with a 0.2-μm membrane for sterility. Control cells for all experiments were treated with deionized distilled water filtered for sterility.

Cell growth assays. Cells were seeded in 96-well plates at a density of 5000 cells/well in a final volume of 100 μl. Twenty-four hours after seeding, the growth medium was replaced with fresh medium containing the vehicle control (sterile dH_20) or increasing concentrations of *Rauwolfia* from 100 to 500 μg/ml. Eight wells were prepared for each *Rauwolfia* concentration and time-point (24, 48 and 72 h). The WST-1 cell proliferation assay (Roche Diagnostics, Indianapolis, IN) was conducted as described by the manufacturer. This assay measures overall mitochondrial dehydrogenase activity in a cell population which correlates to the number of metabolically active cells in the culture.

Flow cytometric analysis of cell cycle and cell death. Following exposure to *Rauwolfia* extract (100, 250 or 500 μg/ml) for 24 h, cells were collected, washed in PBS and fixed in a 2:1 (vol/ vol) solution of chilled ethanol overnight before staining with propidium iodide (PI) in the presence of 100 μg/ml RNAse (DNAse-free). Cell cycle distribution was analyzed for 10000 cells per condition on a Becton-Dickinson flow cytometer (San Jose, CA) and data analysis was performed using 'CellQuest PRO' analysis software. The percentage of total cells partitioning out in Sub-G_0 (sub-genomic DNA) fraction was considered the dead cell fraction.

Detection of PARP cleavage. Cells were treated with 100, 250 or 500 μg/ml of *Rauwolfia* for 24 h, and then collected and processed using standard Western blotting techniques. The polyvinylidene difluoride membranes were probed overnight at 4˚C with anti-PARP antibody (Roche Applied Sciences; Indianapolis, IN) at a dilution of 1/2000 in blocking buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.3% Tween-20 and 5% non-fat dry milk). Goat anti-rabbit IgG HRP (Santa Cruz Biotechnology, Santa Cruz, CA) was utilized as the secondary antibody at a dilution of 1/4000 in blocking buffer. Luminol reagent (Santa Cruz Biotechnology) was used for color development.

Pathway specific gene expression analyses. The effect of the *Rauwolfia* extract on gene expression in LNCaP cells was determined by using pathway specific oligo-based microarray analyses (Oligo GEArrays®, SuperArray Bioscience Corp., Frederick, MD). The microarrays profiled the effect of the extract on the expression of 225 genes involved in human cell cycle (112 genes) and DNA damage (113 genes) signaling pathways. To conduct the microarray analyses, RNA was extracted from both *Rauwolfia*-treated (500 μg/ml, 24 h) and control cells using the Qiagen RNeasy® mini kit as described by the manufacturer (Valencia, CA). RNA was converted to labeled (dUTP-biotin) target cRNA probes and then hybridized to the array membranes according to the manufacturer's protocol (SuperArray Bioscience Corp.). Detection of hybridized probes was conducted using CDP-Star chemiluminescence substrate (SuperArray) following incubation of membranes with alkaline phosphatase-conjuated streptavidin. The resulting signals were scanned and data was analyzed with the manufacturer's software, GEA Expression Analysis Suit. Raw data were normalized against housekeeping genes and the level of significance was set at a 2-fold change in gene expression levels between the control and *Rauwolfia*-treated cells. The upper level of background was set at 2 x readout of blank spots. Each membrane included the following controls: 4 house-keeping genes (6 spots), 2 blanks and 3 negative reference spots. Detailed gene lists of each microarray are available on the company's website (http://www.superarray.com).

Semi-quantitative real-time RT-PCR analysis was carried out on a Roche LightCycler® using the LightCycler-FastStart DNA Master^{PLUS} SYBR Green I kit (Roche) to confirm alterations observed in the gene expression analyses. Commercially available, validated sequence specific primers were utilized for the real-time RT-PCR analyses (RT^2 Real-Time™ gene expression assay kit; SuperArray Bioscience Corp.). Cycling conditions were as follows: 95˚C for 15 min, followed by 40 cycles of 95˚C for 30 sec, 55˚C for 30 sec, and 72˚C for 30 sec. A melting curve analysis was conducted using the following temperature gradient: 95˚C for 0 sec (20˚C/sec), 65˚C for 15 sec (20˚C/sec), and 95˚C for 0 sec with a 0.1-temperature transition rate. RT-PCR GAPDH mRNA was performed in parallel as an internal standard (6). Three independent reactions were run from the same RT sample.

Tumor xenograft generation in nude mice and Rauwolfia treatment. Age-matched male athymic nude mice (Harlan Inc., Indianapolis, IN; 4-5 weeks old) were housed in the barrier facility at Columbia University Medical Center. Mice were randomized into four groups of 10 mice each. LNCaP tumor xenografts were implanted into the right flank on day 1 of the study. For each xenograft, a total of $1x10^6$ cells were suspended in a final volume of 0.5 ml of a 50% suspension in Matrigel (Collaborative Biomedical products, Becton-Dickinson, Bedford, MA) using established procedures (7). Forty-eight hours following tumor xenograft implantation, the mice began receiving daily gavage (0.5 ml) as follows: i) control group (sterilized dH_2O), ii) 7.5 mg/kg *Rauwolfia*, iii) 37.5 mg/kg *Rauwolfia*, or iv) 75 mg/kg *Rauwolfia*. The gavage was performed six days per week at the same time each day for the course of the study (5.5 weeks). Mouse weights were recorded

weekly, and tumor volumes were calculated 2-3 times per week using caliper measurements of length, width and depth (volume = πxh (h^2+3a^2)/6, a = (L+W)/4 (8). On the last day of the experiment, the mice were injected intraperitoneally with 0.5 ml of a 10 mM solution of bromodeoxyuridine (BrdU assay kit, Roche) 4.5 h before being humanely euthanized. Their tumors were removed, fixed in 10% formalin solution for 24 h, and paraffin-embedded.

Immunohistochemical detection of tumor cell proliferation and apoptosis. Paraffin-embedded thin sections of formalin-fixed tumors were de-waxed, rehydrated and stained for BrdU immunoreactivity using an *in situ* cell proliferation kit (POD Kit II, Roche). Sections were counterstained with methyl green (Vector Laboratories, Burlingame, CA). Additional sections were immunostained using an *in situ* cell death detection kit (POD TUNEL assay, Roche). Sections were counterstained with Harris hematoxylin (Sigma, St. Louis, MO). All slides were mounted with cover slips using VectaMount™ (Vector Laboratories).

Statistical evaluation of data. All numerical data were expressed as the average of the values obtained for each group per time-point and error bars shown represent ± standard error of the mean (SEM). For growth curve and flow cytometric analyses, the Student's t-test was utilized to determine statistical significance between each *Rauwolfia* dose group and the control with the level of significance set at $p<0.05$. For the *in vivo* tumor xenograft studies, a Kruskal-Wallis test was conducted to determine if mean tumor volumes differed between the control and *Rauwolfia*-treated groups, and a linear mixed model was then employed to model tumor volume as a function of time and treatment. The p-value was considered significant at $p<0.05$. For immunohistochemical analyses of tumor sections, proliferation and apoptotic indices were determined by light microscopic evaluation (x200 magnification) of immunostained sections. For all mice, three slides per tumor were imaged using a SPOT insight color digital camera and analysis software (Diagnostic Instruments). Two fields were randomly selected on each slide for counting BrdU immunoreactivity and TUNEL staining. Labeling indices for both BrdU and TUNEL analyses were determined by averaging the percent positive staining nuclei per total cells counted (9). Mean and standard error of the mean (SEM) were used to describe labeling indices. Statistical significance between labeling indices of control and *Rauwolfia*-treated mice receiving identical tumor cell xenografts were then determined using the Student's t-test, with the level of significance set at $p<0.05$.

Results

Rauwolfia extract inhibited cell growth and induced apoptosis in LNCaP cells. As shown in Fig. 1, LNCaP cells treated with *Rauwolfia* extract demonstrated a dose-dependent growth inhibition over a 72 h period. While all three doses reduced cell growth in a statistically significant manner by 72 h ($p<0.0001$, Student's t-test), the highest concentration tested, 500 μg/ml, elicited a marked reduction in cell growth from 24 h onward. To further probe the effects of the extract on cell growth, cell cycle analysis revealed that the *Rauwolfia*

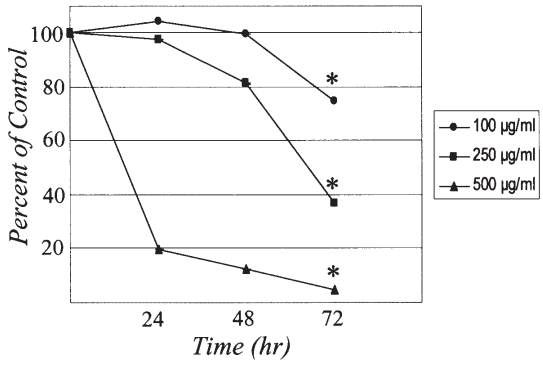


Figure 1. *Rauwolfia* extract reduces LNCaP cell growth over 72 h. Following 24-, 48- or 72-h incubation with the Rauwolfia extract (100, 250 and 500 μg/ml), cells grown in a 96-well format were pulsed for 3 h with WST-1 reagent and absorbances were measured at 450 nM. Values are expressed as means ± SEM (n=8). *$p\leq0.0001$, compared with control cells using the Student's t-test.

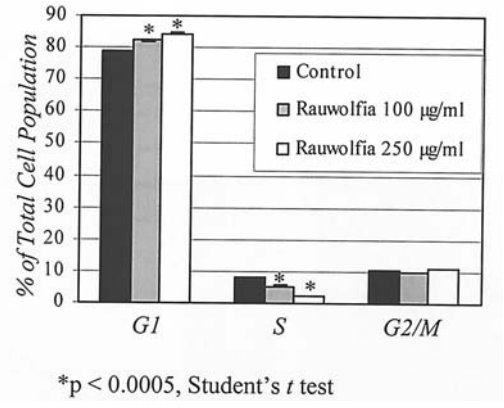


Figure 2. Effects of *Rauwolfia* extract on cell cycle progression. LNCaP cells were exposed to 100 and 250 μg/ml *Rauwolfia* extract for 24 h. Floating and adherent cells were then collected and fixed in a 2:1 ratio (v/v) chilled ethanol for 2 h before staining with propidium iodide in the presence of RNAse. Cells were then analyzed by flow cytometry. Data analysis was performed using CellQuest PRO software. Percent of cells from treated populations that partitioned out into each cell cycle phase were compared to control populations using the Student's t-test (*$p<0.0005$). Each condition was repeated in triplicate.

extract significantly impeded G1 to S phase progression (Fig. 2; $p<0.0005$, Student's t-test), and a nearly 4-fold increase was observed in the G1/S ratio (Table I).

To determine if induction of apoptosis contributed to the reduction in overall metabolic activity of the cell population, treated cell lysates were analyzed for the presence of cleaved PARP by Western blot analysis. By this method, PARP cleavage was only observed following a 24 h treatment of the cells with the highest concentration of *Rauwolfia*, 500 μg/ml, not after treatment with the 100 and 250 μg/ml doses (Fig. 3a). For a more quantitative analysis of the induction of apoptosis, the amount of sub-genomic (Sub-G_0) DNA present was quantified by flow cytometric analysis following propidium iodide staining. This study confirmed the increase in cell death following treatment with 500 μg/ml *Rauwolfia* in a statistically significant manner ($p<0.005$; Student's t-test) when compared to the control cells as shown in Fig. 3b.

Table I. Cell cycle analysis of LNCaP cells treated with *Rauwolfia* extract for 24 h.

Rauwolfia extract	G_1	S	G_2/M	Ratio G_1/S	Ratio S/G_2 M
0 μg/ml	78.71±0.14	8.12±0.01	10.77±0.27	9.69	0.75
100 μg/ml	82.30±0.15[a]	5.44±0.05[a]	10.14±0.37	15.13	0.54
250 μg/ml	84.46±0.09[a]	2.29±0.08[a]	10.93±0.10	36.87	0.21

[a]p<0.0005, Student's t-test.

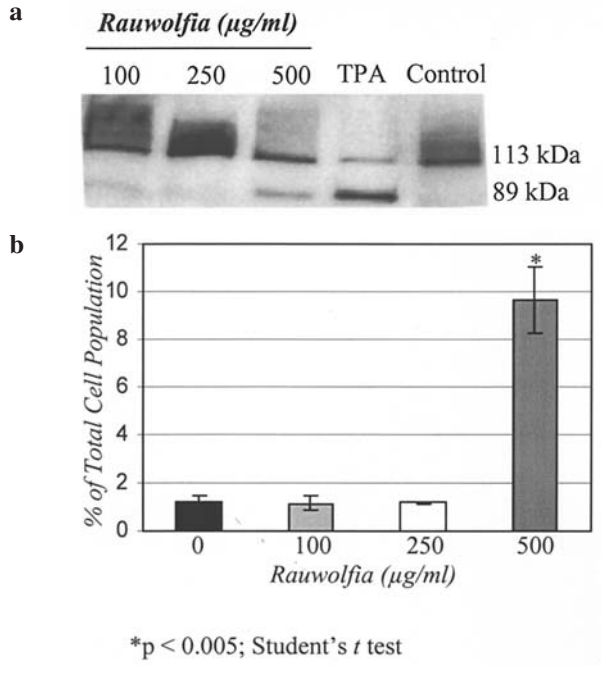


Figure 3. *Rauwolfia* extract induced apoptosis in LNCaP cells, but only at the highest concentration tested (500 μg/ml). (a) Cellular lysates from LNCaP cells that were treated with increasing concentrations of *Rauwolfia* extract for 24 h were processed by Western blotting techniques and probed with anti-PARP antibody. Apoptosis, as measured by the presence of cleaved PARP, was only detected in the samples derived from cells treated with 500 μg/ml of the extract. The experiment was repeated in duplicate. (b) Following treatment of LNCaP cells with the extract, cells were fixed and stained with propidium iodide. Cells were then analyzed by flow cytometry to determine the percentage of the cell population containing sub-genomic DNA. Data analysis was performed using CellQuest PRO software. Each condition was repeated in triplicate and compared to control populations using the Student's t-test (*p<0.005).

Neither the 100 nor 250 μg/ml doses of *Rauwolfia* induced significant accumulation of cells in Sub-G_0, indicating that the extract is only effective at inducing cell death at the highest concentration tested (500 μg/ml).

Rauwolfia extract modulated the expression of several genes involved in cell cycle regulation and DNA damage signaling pathways. Following a 24 h exposure to 500 μg/ml *Rauwolfia* extract, the expression levels of several genes involved in cell cycle control and DNA damage signaling were found to be significantly modified as determined by the pathway specific microarray analyses. The expression levels of seven out of 112 genes included on the cell cycle array were altered significantly (Table II), including markedly reduced expression of both cyclin D1 and E2F and induced p21 expression, thereby supporting an inhibitory action of *Rauwolfia* on G_1 to S phase transition. Cyclin D1 and E2F are known to be involved in G_1 phase and G_1/S transition, while p21 is an inhibitory protein that can prevent cell cycle progression from G_1 to S phase.

The *Rauwolfia* extract appeared to have a greater effect on the expression of the DNA damage signaling pathway genes, as the expression of 49 out of the 113 genes analyzed was altered in a significant manner. Notably, GADD153 expression was elevated to the greatest extent (27.36-fold) following exposure of the LNCaP cells to the test extract, and this gene product is involved in the induction of cell cycle arrest following DNA damage (10). Genes involved in damaged DNA binding and mismatch repair processes comprised the majority of genes with alterations in expression levels >10-fold, including PMS1, NABP2, XRCC3, RAD1, GTF2H3 and FANCG. Details of all *Rauwolfia*-mediated effects on the DNA damage signaling pathway are shown in Table II. These data suggest that through modulation of both DNA damage signaling and cell cycle control pathways, *Rauwolfia* induces cell cycle arrest, apoptosis and DNA repair in the LNCaP cells.

To confirm the results of the microarray analyses by an independent method, the expression of at least two genes from each pathway was determined by semi-quantitative real-time RT-PCR prior to and following the treatment of LNCaP cells with the *Rauwolfia* extract. *Rauwolfia* extract elevated p21 expression 13.74-fold over control levels and reduced cyclin D1 and E2F expression by 0.49 and 0.39, respectively. Additionally, a 27.67-fold increase in GADD153 expression and a 2.31-fold increase in MDG expression were observed. These alterations in gene expression for both cell cycle and DNA damage pathway-specific genes were consistent with the microarray data.

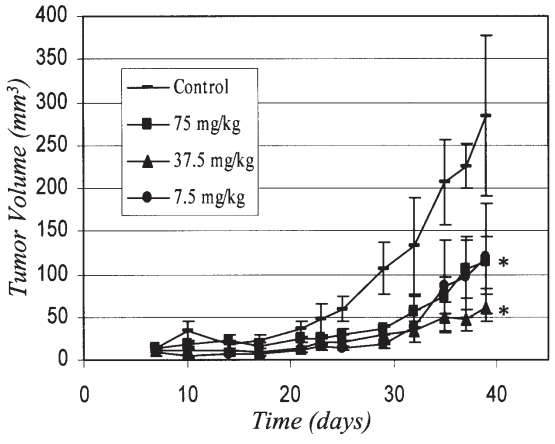
Daily gavage of Rauwolfia extract suppressed the growth of LNCaP tumor xenografts in immunodeficient mice. We utilized an LNCaP tumor xenograft model in immunodeficient mice to determine if a daily gavage protocol with *Rauwolfia* extract could effect tumor growth. Xenografted mice were dosed with the extract daily (6 days/week) by gavage to insure that all mice received equivalent doses. During the course of the study, none of the mice appeared to suffer any

Table II. LNCaP gene expression altered by *Rauwolfia* treatment (500 μg/ml, 24 h).

Human cell cycle microarray Gene	Fold change	Functional gene groupings
p21/Waf1	4.26	Cell cycle check-point and arrest
Cyclin A1	3.18	G_2 and G_2/M transition; regulation of cell cycle
Cyclin A2	2.39	Cell cycle check-point; regulation of cell cycle
MCM2	0.49	S phase and G_1/S transition
MAD2L2	0.47	Cell cycle check-point and arrest
E2F	0.42	G_1 and G_1/S transition; regulation of cell cycle
Cyclin D1	0.14	G_1 and G_1/S transition; regulation of cell cycle
Human DNA damage signaling pathway microarray Gene	**Fold change**	**Functional gene groupings**
GADD153/CHOP	27.36	Cell cycle genes - cell cycle arrest
PMS1	22.40	DNA repair - mismatch repair
N4BP2	22.15	DNA repair - mismatch repair; damaged DNA binding
DNA ligase III	17.80	DNA repair
XRCC3	14.66	DNA repair - damaged DNA binding
RAD1	13.09	DNA repair - damaged-DNA binding; cell cycle genes - cell cycle check-point
GTF2H3	12.21	DNA repair - damaged DNA binding
FANCG	12.16	DNA repair - damaged DNA binding; cell cycle check-point
AIF	11.20	Apoptosis
MYH	8.92	DNA repair - mismatch repair; base-excision repair
MDG	8.83	DNA repair - damaged DNA binding
RAD54	7.83	DNA repair
MRE11A	7.83	DNA repair - double-strand break repair
PMS2	7.22	DNA repair - mismatch repair
RAD51D	6.59	DNA repair - damaged DNA binding
LIG4	6.07	DNA repair
RAD17	5.75	Cell cycle genes - cell cycle arrest
NTH1	5.58	DNA repair - base-excision repair
RAD18	5.15	DNA repair - damaged DNA binding
RAD51C	5.07	DNA repair - damaged DNA binding
HHR23A	4.85	DNA repair
ERCC1	4.63	DNA repair - damaged DNA binding
MNAT1	4.60	DNA repair
RAD51	4.47	DNA repair - damaged DNA binding
GADD34	4.20	Apoptosis; cell cycle arrest
MCG10	4.15	Cell cycle genes - cell cycle arrest
KUB3	4.14	DNA repair - double-strand break repair
Hus1	4.09	Cell cycle genes - cell cycle arrest
MSH2	3.98	DNA repair - mismatch repair; damaged DNA binding
Nibrin	3.95	DNA repair - damaged DNA binding; double-strand break repair
SEMA4A	3.63	DNA repair - damaged DNA binding
XRCC4	3.60	DNA repair - double-strand break repair
PMS2L9, PMS2L3	3.52	DNA repair - mismatch repair; damaged DNA binding
PNKP	3.48	DNA repair - damaged DNA binding
PMS6	3.27	DNA repair - mismatch repair; damaged DNA binding
p73	2.99	Apoptosis; DNA repair - mismatch repair
MSH6	2.99	DNA repair - mismatch repair; damaged DNA binding
TREX2	2.69	DNA repair
RPA3	2.69	DNA repair

Table II. Continued.

Human DNA damage signaling pathway microarray Gene	Fold change	Functional gene groupings
SHIP2	2.48	DNA repair
EXO1	2.34	DNA repair - mismatch repair
RPA1	2.27	DNA repair
XPG	2.20	DNA repair
DNA ligase 1	2.20	DNA repair
MLH3	2.06	DNA repair - mismatch repair
DMC1	2.05	DNA repair - damaged DNA binding
p53	0.47	Apoptosis; cell cycle gene - cell cycle check-point
B99	0.34	Cell cycle genes - cell cycle arrest
ATM	0.32	DNA repair
APEX/Ref-1	0.10	DNA repair - base-excision repair
Cyclin H	0.09	DNA repair



*p < 0.001, Kruskal-Wallis test

Figure 4. Daily feeding of *Rauwolfia* extract significantly reduced growth of LNCaP tumor xenografts in nude mice. For each experiment, 10^6 tumor cells were injected subcutaneously into the right flank of the mice. Animals were fed daily by gavage for 5.5 weeks with the *Rauwolfia* extract (7.5, 37.5 or 75 mg/kg) or sterile dH_2O as the vehicle control. Tumor volumes were measured 2-3 times per week. Mean tumor volume of each group is plotted against time (weeks), and error bars represent ± SEM (n=10). *p<0.001, Kruskal-Wallis test.

untoward toxicity from the *Rauwolfia* extract as assessed by weight gain, feeding behavior and free movement, and none of the control or treated mice died. At the termination of the experiment, all three doses of the *Rauwolfia* extract tested significantly reduced tumor volume with a maximal reduction of 79% occurring in the group of mice receiving the mid-range dose of 37.5 mg/kg (Fig. 4). A Kruskal-Wallis test determined that the control and all three dose groups were significantly different (p<0.001) by the end of the study. To investigate the relationship between the response logarithm of tumor volume and the explanatory variables time and treatment, we used a linear mixed effects model. The random effect was subject, and the fixed effects were treatment and time. We

Table III. Mixed effects linear progression model.

Random effects	SD		
Intercept	0.2853		
Slope	0.0146		
Residual	0.2782		
Fixed effects	**Estimate**	**SE**	**p-value**
Intercept	0.7746	0.0867	<0.0001
Time	0.0414	0.0044	<0.0001
Treatment	0.0266	0.0461	0.5682

fitted both random slopes and random intercepts for each subject, but the variation due to differences in slope was very small compared to the variation due to differences in intercept (Table III). The table also shows that time is a significant predictor (p<0.0001) of the log volume, whereas treatment is not (p=0.5682). However, time interacts significantly with treatment (p=0.00014), so that the overall model should contain both treatment, time and the time-treatment interaction as significant predictors.

All mice received an intraperitoneal bolus of BrdU solution (0.74 mg) 4 h before sacrifice and tumors were recovered on sacrifice, fixed and then embedded prior to sectioning. Immunohistochemical detection of incorporated BrdU was analyzed in all tumors from both control and treated groups to determine the effects of the *Rauwolfia* extract on LNCaP tumor cell proliferation. These data indicate that the *Rauwolfia* extract significantly reduced tumor cell proliferation in all three dose groups (7.5, 37.5 and 75 mg/kg) by approximately 90% (p<0.0001) (Fig. 5a and b). Since all three dose groups induced a statistically equivalent and marked reduction in tumor volume, no dose response was observed.

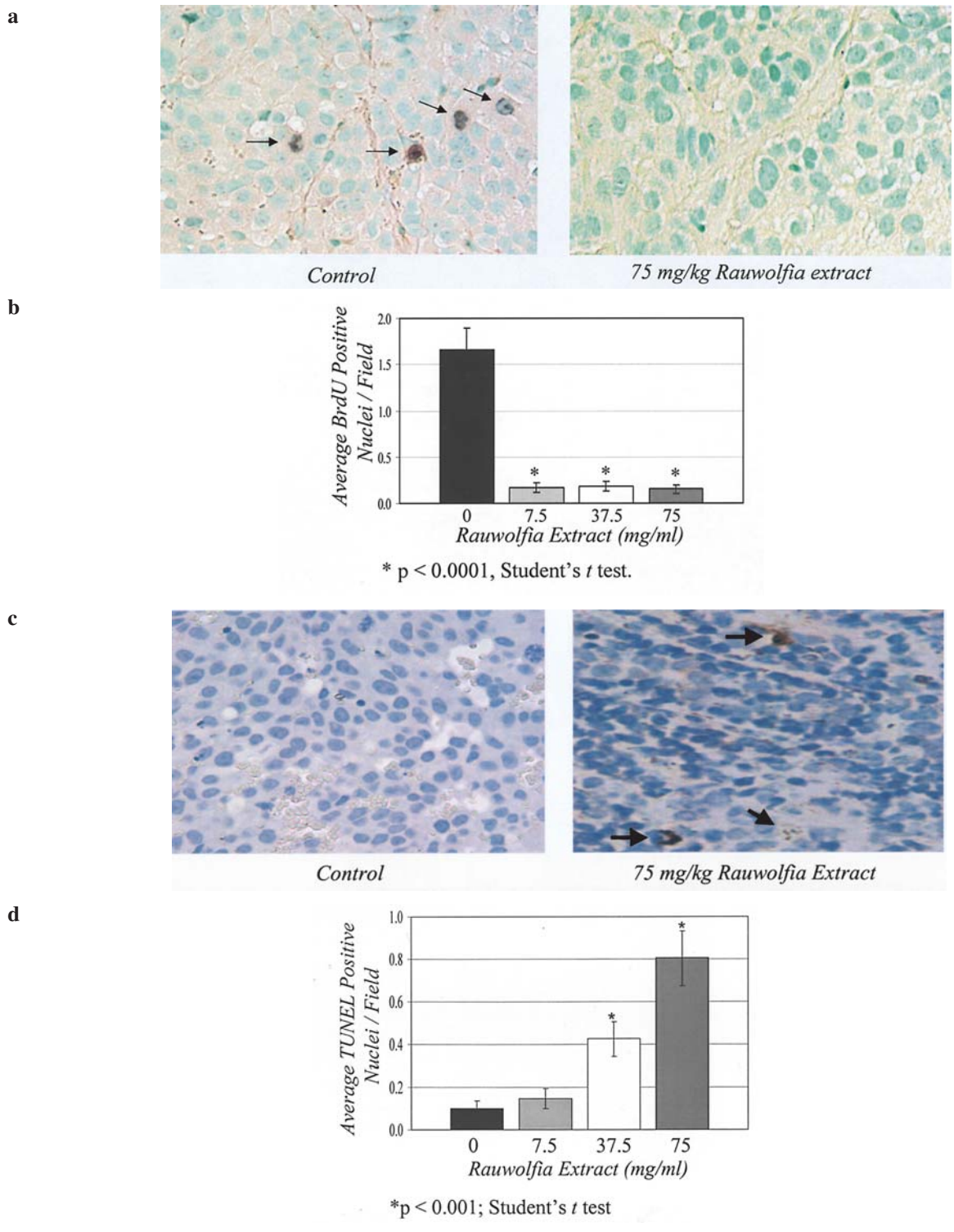


Figure 5. Immunohistochemical detection of BrdU (a and b) and TUNEL (c and d) positive nuclei as measures of proliferation and apoptosis, respectively, in LNCaP tumor xenografts. Tumors were recovered from host mice gavaged daily with *Rauwolfia* extract (7.5, 37.5 or 75 mg/kg) or sterile dH_2O as a vehicle control and were immunostained for BrdU or TUNEL reactivity. Peroxidase-stained BrdU and TUNEL positive nuclei were counted after counterstaining with methyl green or Harris hematoxylin, respectively (x400 magnification, arrows indicate BrdU and TUNEL positive nuclei). The average number of immunopositive nuclei per each treatment group was compared to controls using the Student's t-test. For BrdU staining, $^{*}p<0.0001$; and for TUNEL assay, $^{*}p<0.001$.

Furthermore, to determine if the reduction in overall tumor volume observed in *Rauwolfia*-treated groups was in part due to induction of apoptosis, tumor sections were analyzed for TUNEL positive nuclei. While no difference in tumor cell apoptosis was observed between the low-dose group (7.5 mg/kg) and the control group (p=0.441), statistically significant increases in tumor cell apoptosis of 4.26-fold in the 37.5 mg/kg (p<0.0005) and 8.03-fold in the 75 mg/kg (p<0.0001) groups were seen (Fig. 5c and d). Taken together with the tumor volume measurements and the tumor cell proliferation studies,

it is interesting to note that the low-dose group (7.5 mg/kg) reduced tumor burden in the mice to an equivalent level as the 37.5 and 75 mg/kg dose groups and this action appeared to be mainly a function of the extract's effects on tumor cell proliferation rather than induction of apoptosis.

Discussion

Deaths attributable to prostate cancer are among the highest of all tumor types in men and treatment success rates of advanced disease remain low (1,2,11). These facts, in addition to a long latency period of up to 20-30 years prior to development of metastatic disease, identify this form of cancer as an ideal target for chemoprevention and/or early intervention. However, treatment with a chemotherapeutic agent in the context of preventing the development of disease is clearly not an acceptable course of therapy because of the general toxic nature of the agents used for this purpose. Due to the preponderance of bioactive compounds in certain plant and herbal mixtures, it is conceivable that such preparations could potentially offer therapeutic benefits in these patients if efficacy and lack of toxicity are demonstrated. A general concept put forward by several researchers is that the anti-cancer activity of the compounds which are typically present in plants at sub-pharmaceutical doses could synergize to delay or disrupt the development of aggressive disease (12,13). With these goals in mind, this study is the first step in the identification of potential anti-cancer activity of an extract derived from a medicinal plant, *Rauwolfia vomitoria*, historically used by indigenous people of sub-Saharan Africa.

The data presented herein has shown that the *Rauwolfia* extract is an effective inhibitor of cell growth in the human prostate cancer cell line, LNCaP, in both cell culture and *in vivo* tumor xenograft experimental systems. Microarray analyses point towards the effects of the extract on both DNA damage and cell cycle control pathways. Although higher doses of the *Rauwolfia* extract resulted in induction of apoptosis in both *in vitro* and *in vivo* settings, this response does not appear to be the primary mechanism of action through which the extract impacts overall tumor volume *in vivo*. Comparison of the low- and high-dose *Rauwolfia* groups revealed no difference in overall tumor volumes while the number of cells undergoing apoptosis was significantly greater in the tumor xenografts receiving the high dose of the extract. These data suggest that the main mechanism through which the *Rauwolfia* extract impacts overall tumor volume is by reducing tumor cell proliferation.

As cancer cells often have mutations in genes regulating DNA damage responses or repair pathways, they can be more susceptible to cell cycle arrest and death from treatment with genotoxic agents than normal cells (14). Many chemotherapeutic agents, including plant-derived compounds such as etoposide and the vinca alkaloids, exert their toxicity by directly interacting with DNA or DNA-binding proteins (15,14). This interaction triggers DNA damage signaling pathways resulting in inhibition of cell proliferation or induction of apoptosis, depending on the extent of the damage (16). Following treatment of LNCaP cells with the *Rauwolfia* extract, microarray analyses identified a profound effect on genes associated with DNA damage signaling, in particular those that code for proteins which bind selectively to damaged DNA as part of the DNA repair process. This response suggests that the extract induces a DNA damage response which is large enough in magnitude to prevent the prostate cancer cell from recovering, thereby resulting in cell cycle arrest.

The most highly modified gene following treatment with the *Rauwolfia* extract was GADD153, which is known to be induced by genotoxic stress and encodes for proteins with anti-proliferative activity (17). Overexpression of this protein has been shown to induce cell cycle block, as well as apoptosis (18,19). DNA damage or genotoxic stress generally result in an interruption in cell cycle progression at G_1 and G_2 checkpoints, and this is thought to occur to allow time for DNA repair. We also observed an attenuation of G_1 to S transition following LNCaP exposure to the *Rauwolfia* extract which correlates well with the induction of DNA damage signaling pathway.

Although the p53 protein is often thought of as a main mediator of DNA damage signaling, we note that p53 expression did not change significantly following LNCaP exposure to the extract. However, p53 protein levels in the cell are mainly regulated by post-translational modification, and therefore mRNA levels are not correlative to protein levels within the cell (20). Further analysis of p53 protein levels needs to be conducted to determine if the extract increases the stability of this protein. It is important to note that expression of p21, which is the main p53 target gene, was found to be significantly increased. p21 is a cyclin-dependent kinase inhibitor and can inhibit CDK2 leading to G_1 arrest and CDK1 inducing G_2/M phase arrest.

Interestingly, the *Rauwolfia* extract appeared to have opposing effects on the expression of several cyclins. A significant increase in expression of both cyclins A1 and A2 was observed, while expression of cyclins D1 and H were dramatically reduced. Cyclins are known to be part of the main regulatory machinery that directs transition through phases of the cell cycle and their expression levels have been characterized to oscillate in synchrony with the cell cycle. Other functions of several cyclins have been more recently described, including roles for cyclin A and H in DNA damage signaling (21,22). Expression of cyclin A1 is generally tissue specific and restricted to the testis in adults, however induction of the protein has been reported in both HuTu80 colon carcinoma cells and in mice in response to γ-irradiation as part of the cellular response to DNA damage (21). Cyclin A1 expression was also reported to correlate to tumor aggressiveness in human prostate tumors, and its induction in LNCaP cells appeared to be involved in the mediation of elevated VEGF following exposure of the cells to synthetic androgen (23). Cyclin A2 is present in proliferating somatic cells and regulates the progression through S phase and mitosis (24). The significance of the increase in cyclin A1 and A2 expression in LNCaP cells following *Rauwolfia* is still unclear.

The largest reductions in gene expression were observed in both cyclins D1 and H. Cyclin D1 is well known for its association with cdk4/6 and regulation of G_1 progression. Cyclin H comprises part of the CAK complex (cdk activating kinase), which phosphorylates cdks resulting in their activation (25). CAK kinase activity has been previously shown to be reduced after UV light irradiation (26) and this down-regulation

of kinase activity was subsequently determined to be mediated by direct association of the cyclin H component with p53 (27). Although the direct mechanism of action involved cannot yet be explained, the down-regulation of both cyclin D1 and H observed in the LNCaP cells following exposure to the *Rauwolfia* extract is consistent with a growth inhibitory response in the cells.

While further experiments need to be performed with this extract to uncover the exact molecular targets involved, initial results indicate that the growth suppression of human prostate cancer cells *in vitro* and *in vivo* involves the activation of DNA damage signaling pathway and suppression of transit through the cell cycle. As this preparation contains a complex mixture of natural compounds, the potential to impact multiple molecular targets could prove useful for a chemopreventive agent if no appreciable toxicity exists.

The ultimate goal of this study is to determine if this extract may have clinical utility in human patients. To that extent, we are currently conducting a phase I clinical study to examine the potential toxicity of this extract in combination with another ß-carboline alkaloid enriched plant derived from the bark of the Pao Pereira tree in patients with elevated PSA and no clinical signs of prostate cancer based on negative biopsy reports. Secondary end-points of this study will consider the effects on PSA levels over the 12-month course of the study. This important first step will allow us to determine if this extract preparation will be a potential candidate for a chemopreventive agent.

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