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Potato disc tumor induction assay: A multiple mode of drug action assay

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Summary

The study reported herein utilized the *Agrobacterium tumefaciens*-induced potato disc tumor assay. The objective was to verify the detection of antineoplastic activity in the potato disc tumor induction assay, regardless of the mode of antineoplastic drug action. Camptothecin, paclitaxel, podophyllin, vinblastine and vincristine were tested, each with a different mode of action. All drugs tested inhibited tumor induction. The order of activity was: camptothecin = paclitaxel = vinblastine < podophyllin = vincristine. No effect on the viability of the bacterium was detected. The *A. tumefaciens*-induced potato disc tumor assay was an effective indicator of antitumor activity regardless of the mechanism of drug action. Thus, this assay would be acceptable as a primary general screen for antineoplastic activity of various crude extracts, as well as for purified fractions, regardless of mode of inhibitory action on tumor formation.

Key words: Screening for antineoplastic activity, *Agrobacterium tumefaciens* tumor indiction assay

Introduction

Traditional medicine based on folklore and anecdotal information has produced leads for new antitumor and antibiotic drugs (Wedge and Camper, 1999). *Vinca rosea* was traditionally used for diabetes and was found to decrease white blood cell counts (Lippincott and Lippincott, 1975). *Vinca* alkaloids are used to treat pediatric leukemia and other neoplasms today. *Podophyllum peltatum* was used by the Penobscot Indians to treat cancer. Others used the resin to remove venereal warts, and it is still the treatment of choice (Gordaliza et al., 1994). Derivatives of *Podophyllum* are used today to treat small lung and testicular cancer. Native American Indians used *Taxus* spp. as an abortifacient as well as for skin cancers. Taxol from *Taxus brevifolia* is used for ovarian and breast cancer. The development of a pharmaceutical of ethnobotanical, anecdotal or folkloric origin can be expensive and require years of experimentation. Verification of claimed biological activity requires testing in bioassays and ultimately clinical testing. Bioassays can provide information about the biological activity of a plant extract and can also be used to direct fractionation of the extract to identify active components.

Bioassay methods used in assessing the antitumor activity of plant extracts have varied over the years. These methods have yielded important discoveries including vincristine, vinblastine, the podophyllotoxin derivatives, 10-hydroxy-camptothecin and Taxol (Suffness and Douors, 1979; Wani et al., 1980). The 3PS (P388) (methylcholanthrene-induced) leukemic mouse assay and the *in vitro* screening for 9KB (human nasopharyngeal carcinoma) cytotoxicity (Jackson et al., 1984; Wall and Wani, 1977) came to be the assays of choice. New primary *in vitro* screening assays were developed by the Developmental Therapeutics program of National Cancer Institute (NCI) and now consists of a panel of over 50 human cancer cell lines (McLaughlin and Rogers, 1998). These assays identify agents having cell-type selective toxicity (Paclitaxel). However, they are costly, require special-

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ized laboratories and equipment, and may present problems in interpreting data obtained with plant extracts.

The inhibition of *A. tumefaciens*-induced tumors (or Crown Gall) in potato disc tissue is an assay based on antimitotic activity and can detect a broad range of known and novel antitumor effects (McLaughlin and Rogers, 1998). Crown Gall is a neoplastic plant disease caused by *A. tumefaciens* (Kahl and Schell, 1982; Lippincott and Lippincott, 1975). The validity of this bioassay is predicted on the observation that certain tumorigenic mechanisms are similar in plants and animals (Becker, 1975; Braun, 1972; Karpas, 1982). It has been shown that the inhibition of Crown Gall tumor initiation on potato discs and subsequent growth showed good correlation with compounds and extracts active in the 3PS leukemic mouse assay (Galsky et al., 1980; Galsky et al., 1981). Ferrigni et al. (1982) showed that the potato disc tumor assay was statistically more predictive of 3PS activity than either the 9KB or the 9PS cytotoxicity assays. Podophyllin, Taxol, Camptothecin, Vincristine and Vinblastine have all shown significant tumor inhibition in the 3PS (also referred to as P-388) leukemic mouse assay (Gordaliza et al., 1994; Kahl and Schell, 1982; Lewis and Elvin-Lewis, 1977; Riley, 1999).

The bacterium, *A. tumefaciens*, is a gram-negative rod that is the causative agent of Crown Gall Disease. Crown Gall Disease is a disease in which a mass of tissue bulging from stems and roots of woody and herbaceous plants is produced. These masses (tumors) may be spongy or hard, and may or may not have a deleterious effect on the plant. The tumors produced are histologically similar to those tumors found in humans and animals (Agrios, 1997).

During infection of plant material with *A. tumefaciens*, a tumor-producing plasmid (Ti-plasmid), found in the bacterial DNA, is incorporated into the plant's chromosomal DNA. When plant tissue is wounded it releases phenols, etc., which will activate the Ti plasmid in *A. tumefaciens*. The Ti-plasmid causes the plant's cells to multiply rapidly without going through apoptosis, resulting in tumor formation similar in nucleic acid content and histology to human and animal cancers (Agrios, 1997). McLaughlin (1991) concluded that the Crown Gall tumor (potato disc) assay could be used as a fairly rapid, inexpensive and reliable prescreen for antitumor activity.

The objective of this study was to assess the response of selected plant-derived chemotherapeutic agents with different modes of action in the potato disc tumor assay. Camptothecin, paclitaxel, podophyllin, vinblastine and vincristine are FDA-approved chemotherapeutic drugs originating from plants, and exhibit diverse modes of action on the cell cycle.

Material and Methods

A. tumefacies was grown on Yeast Extract Media (YEM) for 48 h at 28 °C. Russet potatoes (*Solanum uberosum* L.) were disinfested by scrubbing under runing water with a brush, then immersing in 10% Clorox for 20 min. Potatoes were removed from the Clorox, blotted on sterile paper towels, and each side removed allowing for a flat surface without skin. The trimmed sections were placed in Clorox (20%) for 15 min. Cylinders were cut from the disinfested sections using a sterile cork borer (10 mm). Each cylinder segment was placed in sterile distilled water. After rinsing, each end of the cylinder was excised and discarded and the remaining cylinder was rinsed again in sterile distilled water. Disks (0.5-cm thick) were cut aseptically from the cylinders. These disks were placed in a 24-well culture plate containing 15% water agar. Suspensions of *A. tumefaciens* in phosphate-buffered saline (PBS) were standardized to 1×10^9 Colony Forming Units (CFU) as determined by an absorbance value of 0.96 ± 0.02 at 600 nm. Camptothecin, vinblastine, vincristine, podophyllin and paclitaxel (all obtained from Sigma Chemical Co., St. Louis, MO) were dissolved in dimethylsulfoxide (DMSO) at a concentration of 1 mg/ml and subsequently diluted to 0.1, 0.01 and 0.001 ng/l. Camptothecin served as a positive inhibitory control (McLaughlin and Rogers, 1998). Other controls included: DMSO with phosphate buffered saline (PBS); DMSO without the bacterium and DMSO with the bacterium. The test solutions consisted of 400 µl of the drug or control solution, 100 µl water, and 400 µl of the standardized bacterium suspension.

Each disk in the 24-well culture plate was overlaid with 50 µl of the appropriate extract/water/bacteria mix and incubated at room temperature for 12 days. On day 12, the disks were stained with Lugol's Reagent (I_2KI; 5% I_2 plus 10% KI in distilled water). Lugol's reagent stains the starch in the potato tissue a dark blue to dark brown color, but the tumors produced by *A. tumefaciens* will not take up the stain, and appear creamy to orange (McLaughlin and Rogers, 1998). The stained potato disks were viewed under a dissecting microscope and the tumors were counted. Twelve replicates were analyzed for each sample, and all experiments were repeated three times. Data was analyzed using the SAS program.

Bacterial viability was determined by incubating each drug (0.1 mg/l) with 1×10^9 colony-forming units (CFU) of bacterial suspension (contained in phosphate buffered solution: 0.043% KH_2PO_4, 0.148% Na_2HPO_4 and 0.72% NaCl; four Eppendorf tubes per test). At 10, 20, 30 and 60 min after inoculation, 10 µl of each solu-

tion was removed and placed on YEM plates, and incubated for 24 h. Bacterial growth was evidenced by growth across the plates.

Results and Discussion

Three internal control treatments were included in this study. *A. tumefaciens* with DMSO induced an average of eight tumors per potato disc. DMSO alone did not induce any tumors. Thus, DMSO as a solvent did not interfere with the activity of the bacterium or induce tumors itself. Camptothecin served as a positive control and inhibited tumor production at all concentrations tested.

There was a significant difference in activity between camptothecin, podophyllin and vincristine at 0.001 ppm; but camptothecin was not significantly different from paclitaxel at the same concentration. Camptothecin was significantly more active at 0.01 ppm than podophyllin, vinblastine or vincristine at the same concentration. No significant difference was observed between camptothecin at 0.1 ppm and podophyllin, vinblastine, or taxol at the same rate. Camptothecin at 0.1 ppm was significantly more inhibiting than vincristine at 0.1 ppm.

Camptothecin and its analogs are aromatic, planar alkaloids; camptothecin was isolated from the fruit of *Camptotheca acuminata* in 1966 by Wall et al. (1966). At that time, it was known to be antitumorigenic, but was abandoned due to its high toxicity. Two camptothecins, topotecan and irinotecan, are FDA approved chemotherapeutic agents for metastatic carcinoma of the colon and rectum, as well as ovarian, cervical, gastric, lung and esophageal cancers. The camptothecins stop cells from completing mitosis, specifically in the S-phase. They inhibit topoisomerase I and break DNA chains during replication which changes the three-dimensional structure of DNA, thereby stopping replication. Dosage range is 25–150 mg/m^2 with an optimum dose of 125 mg/m^2 (Voigt et al., 1998). Our assays utilized concentrations of 0.1, 0.01 and 0.001 ppm.

Vinblastine inhibited tumor production at all concentrations, with that of 0.1 ppm being the most inhibitory. Vinblastine was more inhibitory than vincristine at 0.01 ppm, thus less inhibitory than podophyllin, but not different from paclitaxel and camptothecin. Vinblastine, discovered in 1959, is an alkaloid extracted from *Vinca rosea* (Periwinkle). It was originally investigated for its use in hypoglycemia. When it was found to suppress bone marrow, it was investigated as a possible antitumorigenic agent (Spjut, 1985). Vinblastine (Trade names: Velbe, Velban, VBL Purpose) is an FDA-approved drug for Hodgkin's and Non-Hodgkin's lymphomas, as well as for testicular cancer, choriocarcinoma and Kaposi's Sarcoma. Vinblastine stops the cell cycle between prometaphase and anaphase by altering spindle fibers (binding to microtubules and blocking polymerization into microtubules), preventing further replication. Vinblastine is immunosuppressive, and is usually used in conjunction with other drugs. The therapeutic range for an adult is 3-7–11.1 mg/m^2 and for a child, 2.5–7.5 mg/m^2 (de Lemos, 2001; Rao et al., 1989; Riley, 1999; Wendell et al., 1999). Our assay utilized concentrations of 0.1, 0.01 and 0.001 ppm.

Vincristine significantly inhibited tumor formation at all concentrations tested. Vincristine, discovered in 1959, is an alkaloid extracted from *Vinca rosea* (Periwinkle). It was originally investigated for its use in hypoglycemia. Although the mode of action of Vincristine is not fully understood, it is thought to interfere with tubulin function by binding to microtubules and spindle proteins in the S-phase of the cell cycle, leading to cell death. Vincristine (Trade names: Oncovin, Vincasor) is FDA-approved for Hodgkin's and Non-Hodgkin's lymphoma, Wilm's tumor, and lung cancer. It is sometimes used for Neuroblastoma, brain tumors, and breast and cervical cancers. It is administered by IV only at a dosage range of 1.4–2.0 mg/m^2 (de Lemos, 2001; Riley, 1999; Spjut, 1985). Our assay utilized concentrations of: 0.1, 0.01 and 0.001 ppm.

Podophyllin inhibited tumor formation at all concentrations tested. Podophyllin is a keratolytic resin extracted from the plant, *Podophyllum peltatum* (Mayapple). It was used originally for genital warts, but was found to be effective in stopping mitosis in metaphase, much like the *Vinca* alkaloids. It causes breaks in double-stranded DNA and inhibits topoisomerase II activity (specifically inhibiting release of the enzyme from DNA), leading to cell death. Etoposide (Trade names: Vepesid, VP-16, VP-16-213, EPEG, EPE) is a semisynthetic antineoplastic agent based on podophyllotoxin, and is FDA-approved for testicular and small lung cancer and for Non-Hodgkin's lymphoma. It has been used less frequently for brain tumors, Ewing's sarcoma, Kaposi's sarcoma, Neuroblastoma and ovarian cancer. It is administered by IV, or orally. The adult therapeutic range for IV use is 100–250 mg/m^2 on day 1, with 35–150 mg/m^2 for days 3 through 5. Oral dose range is 150–200 for 5 days, 50 mg/m^2 for 21 days or 50–100 mg for 1 to 2 months. Often IV therapy is administered for 1 day and oral medication continued for 5 days. Children are given 100–150 mg/m^2 IV for 3 to 5 days (de Lemos, 2001; Damayanthi and Lown, 1998; Riley, 1999). Our assay itilized concentrations of 0.1, 0.01 and 0.001 ppm.

Paclitaxel inhibited tumor formation at all concentrations, with no significant difference between the concentrations tested. Paclitaxel was no different from camptothecin at the same concentrations. The National

Table 1. *Agrobacterium*-induced tumor formation on potato discs by different drugs.

Conc. (ppm)	Mean number[1] of tumors produced				
	Campt.	Podophyl.	Taxol.	Vinblast.	Vincrist.
0.001	$0.34^{a,x}$	$0.46^{a,y}$	$0.24^{a,x}$	$0.14^{a,x}$	$0.86^{a,y}$
0.01	$0.13^{a,x}$	$0.68^{a,y}$	$0.09^{a,x}$	$0.28^{a,x}$	$0.49^{a,y}$
0.1	$0.34^{a,x}$	$0.28^{b,x}$	$0.02^{a,x}$	$0.22^{a,x}$	$0.86^{a,y}$
*A. *tumefaciens*	8c	8c	8c	8c	8c
**DMSO	0	0	0	0	0

[1] Numbers represent the average number of tumors produced.
* *Agrobacterium* without drug; tumor initation control.
** DMSO without *Agrobacterium* or plant extract; solvent control.
[a,b,c] Values with the same letters are not different within vertical columns (i.e., concentrations).
[x,y] Values with the same letters are not different within horizontal columns (i.e., drug comparison).

Table 2. Growth (indicated by + or –) of *Agrobacterium tumefaciens* on YEM medium as a function of incubation time (min) in phosphate buffered medium and 0.01 mg/ml each of camptothecin (camp.), podophyllin (podoophyl.), vincristine (vincrist.) and vinblastine (vinblast.). Negative controls (cnt) contained jsut the bacterium inoculum; positive controls contained DMSO with the bacterium inoculum.

Exposure time (min)	Growth					
	Neg. cnt.	Pos. cnt.	Camp.	Podophyl.	Vincrist.	Vinblast.
10	+	+	+	+	+	+
20	+	+	+	+	+	+
30	+	+	+	+	+	+
60	+	+	+	+	+	+

Cancer Institute identified paclitaxel in an extract from the Pacific Yew Tree *(Taxus brevifolia)* in 1962. Anecdotal information from India identified extracts from Yew as anticarcinogenic. The structure (a diterpenoid compound) and mode of action of paclitaxel (Trade Name: Taxol) were elucidated during the 1970's and clinical trials begin in 1983 with subsequent FDA-approval in 1992. Rather than interfere with spindle formation like other chemotherapeutic agents, paclitaxel binds specifically to the β-tubulin subunit of microtubules and appears to antagonize the disassembly of this key cytoskeletal protein, resulting in bundles of microtubules and aberrant structures, and an arrest of mitosis. Derivatives of paclitaxel are used for ovarian and breast cancers as well as for Kaposi's sarcoma. It is administered IV with a therapeutic range of 135–175 mg/m^2 given over 3 hours (de Lemos, 2001; Damayanthi and Lown, 1998; Riley, 1999). Our assay utilized concentrations of: 0.1, 0.01 and 0.001 ppm.

All compounds tested in this study, inhibited tumor formation in the Potato Disc Tumor Assay. Tests showed that the drugs tested herein did not affect the viability of the bacterium (Table 2). Thus, the action of the drugs tested herein is on the formation of tumors and not on the viability of the bacterium. The initial step in the formation of *A. tumefaciens*-induced tumors involves attachment of the bacterium to a tumor-binding site (Glogowski and Galsky, 1978). The attachment of the bacterium to the tissue is complete within 15 min following inoculation (Glogowski and Galsky, 1978; McLaughlin et al., 1993). Galsky et al. (1980) examined the effects of several compounds and plant extracts on Crown Gall tumor formation and found no effects on bacterial viability or on the attachment process. The most active tumor inhibitors were camptothecin and paclitaxel, followed by vinblastine, podophyllin, and vincristine, in that order.

Mechanism-based assays detect potential anticancer agents that interfere with neoplastic growth, or focus on target receptors that were discovered as the mechanism of drug action (McLaughlin, 1991). Crown Gall is a neoplastic disease of plants induced by specific strains of *A. tumefaciens*. Galsky et al. (1980) first demonstrated that inhibition of Crown Gall tumor dis-

tribution on potato discs correlated with compounds and plant extracts known to be active in the 3PS leukemic mouse tumor assay. The antitumor effect is independent of antibiosis (Galsky et al., 1980). Thus, as a prescreen, antibiosis is not a problem. The Potato Disc Tumor Assay is a simple, inexpensive and fast screen for antitumor agents. It was used to identify the antitumor activity of ellagic acid and an extract of *Meliva volkensii* fruit (Wedge and Camper, 1999). The study reported herein affirms the use of this assay as a first general screen in the search for new antitumor agents, whether their mode of action be inhibition of topoisomerase, interference with tubulin function, or prevention of microtubule reorganization.

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