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IN VITRO ANTICANCER STUDIES ON CANTRON® AND ITS CONSTITUENTS

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ABSTRACT: ***The anticancer activity of Cantron® and its presumed constituents were examined in vitro against a number of leukemia, solid tumor and normal cell types in a disk diffusion assay. It was demonstrated to have solid tumor selectivity against murine Colon38 compared to both murine leukemia L1210 and murine bone marrow stem cells (CFU-GM). The cytotoxic compounds in Cantron® with significant anticancer activity in vitro were shown to be catechol and tetrahydroxyquinone (THQ). IC_{50} values were determined against 13 different cancer cell lines and yielded an average value of 22.8 µg/mL for Cantron®, 2.3 µg/mL for catechol and 23.7 µg/mL for THQ. Clonogenic studies for these three materials demonstrated a similar increase in cell killing as a function of exposure time (from 2 h to 7 days) with the 7 day S_{10} value (concentration with yielded a 10% survival of clonogenic cells) of 5, 1.6 and 5 µg/mL, respectively, for Cantron®, catechol and THQ. An HPLC assay for catechol demonstrated its percentage in Cantron® at 12.7% by weight, a value also deduced from the cytotoxic activities of the components of Cantron®. These studies indicate that Cantron® has both anticancer activity and therapeutic potential.***

KEY WORDS: Alternative therapy, Anticancer, Cantron, Protocel

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INTRODUCTION

Alternative, non-FDA approved, therapies for cancer are becoming increasingly common and more acceptable to the general public. This is due in part to the public's frustration with the lack of effective medical treatments for many cancers such as lung, brain and pancreas, the high cost of pharmaceuticals, the significant adverse side effects of anticancer drugs and the abundance of anecdotal evidence both verbally and on the Internet for successful treatments using alternative approaches. The increased use of alternative medicine approaches led to the creation of the National Center for Complementary and Alternative Medicine by the National Institute of Health in the United States to "explore complementary and alternative healing practices in the context of rigorous science" (http://nccam.nih.gov). Given their prevalence in the market place and the public's desire to utilize these alternatives, it is important that the scientific community examine alternative medicines either to validate them or to demonstrate their lack of efficacy.

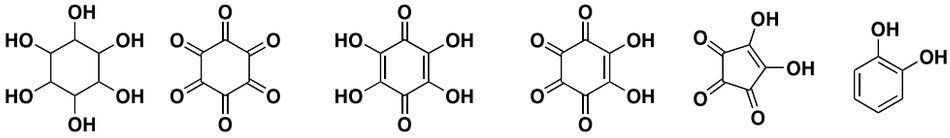
One popular alternative cancer therapy is known as Cantron® (Entelev®, Cancell®, Protocel®, Jim's Juice). Cantron®, in one form or another has been produced since the late 1940's when Jim Sheridan, a chemist working at Dow Chemical and later at the Detroit Institute of Cancer Research (which became the Michigan Cancer Foundation), produced a chemical formulation called Entelev®. He tested it in the 1950's and 1960's and from the early 1970's to 1983 he provided it free of charge to terminally ill cancer patients. Sheridan filed an INDA (IND# 20258) with the FDA in 1982; however, a clinical trial did not proceed due to the lack of animal trial data. Because unapproved claims for the cure of cancer and autoimmune diseases were disseminated, in 1983 the FDA served Sheridan with a cease and desist order. A review of the history of Entelev® has been published by the American Cancer Society (Anonymous, 1993). This article also reviewed the proposed mechanisms of action of Cancell/ Entelev® although none proposed is backed by accepted scientific assessment. Today, an Entelev® surrogate called Cantron®, is manufactured and marketed by Medical Research Products (MRP) in Miami, Florida, described as an antioxidant and electrolyte formula (http://www.cantron.com.). The manufacturer of Cantron® offers it as a dietary supplement and makes no specific

health claims. The first batch of Cantron® was produced in 1984. In 2000, a further formulation of Entelev/Cancell was manufactured and sold under the name Protocel® (Remission and Wellness, LLC., Simpsonville, SC).

Supporters of Cantron® claim that it is able to treat any number of medical conditions from emphysema, arthritis, lupus and mental illness to AIDS and cancer. Because it was originally designed by Jim Sheraton as an anticancer agent and has been used by cancer patients since the 1970's, there is an abundance of anecdotal and testimonial data concerning its cancer efficacy (http://alternativecancer.us/cantron.htm, www.youtube.com, search terms: Cantron Hope, http://www.rationalwellnessgroup.com.).

The ingredients of Cantron®, as written on the product label, are copper, sodium, potassium, sulfur, trace amounts of inositol, distilled water and a "proprietary blend". The label also states that hydroxyquinones and cyclopentenes are present in the product. The FDA determined that Cantron® contains nitric acid, sodium sulfite, potassium hydroxide, sulfuric acid, inositol and catechol. Other reported ingredients in Cantron® are rhodizonic acid (RA), croconic acid (CA), tetrahydroxyquinone (THQ), leuconic acid, and triquinoyl (http://www.cancer.gov/cancertopics/pdq/cam/cancell/healthprofessional/allpages). The structures of these compounds are indicated in Figure 1.

FIGURE 1: Chemical constituents of Cantron®



In the early 1990's the National Cancer Institute examined the anticancer activity of Cancell (NSC 637907) and deemed it to be inactive and not worthy of further study (http://dtp.nci.nih.gov/dtpstandard/cancerscreeningdata/index.jsp (Key words: Cancell, NSC 637907). To date, there have been no published biological or clinical studies demonstrating any anticancer effect of any of the formulations, including Cantron®, making justification of the anticancer claim impossible. However, due to the overwhelming amount of testimonial and anecdotal data and at the request of a Cantron® user who claimed some effect for himself and members of his family, we decided to examine Cantron® in our cancer drug discovery paradigm.

Our drug discovery and development paradigm has been discussed previously in the literature (Subranamian, Nakeff et al, 2006; Valeriote, Media et al., 2012). The paradigm involves a series of *in vitro* and *in vivo* studies accessing the clinical potential of the compound tested. Once a drug has been found to be solid tumor selective in our *in vitro* zone assay, it is moved to early preclinical development where IC_{50} and clonogenic properties are defined *in vitro*. An analytical assay for the compound is followed by intravenous formulation, determination of maximum tolerated dose (MTD) and the pharmacokinetic profile of the drug *in vivo*. Analysis of these values determines whether a compound passes into late preclinical development where a therapeutic assessment is performed. If there is sufficient therapeutic efficacy, Investigational New Drug Application (INDA)-related studies are done leading ultimately to a human clinical trial. In the studies reported here, the initial, *in vitro* assessment is presented.

MATERIALS & METHODS

Compounds

Catechol, tetrahydroxyquinone, rhodizonic acid, croconic acid, inositol, and sodium sulfite were purchased by Sigma-Aldrich (Atlanta, GA). Triquinoyl was prepared in our laboratory by one of us (R.B.). The compounds were solubilized in dimethyl sulfoxide (DMSO) and further diluted in ethanol. Cantron® was kindly provided by Mr. Jerome Godin (Medical Research Products, Inc., Miami).

In Vitro Disk Diffusion Assay

The step-by-step laboratory methods for our *in vitro* assay have been described (Valeriote, Grieshaber et al., 2002). A short outline of that procedure is as follows:

Preparation of cell suspensions**:** A monodispersed cell suspension of Colon 38 is prepared by mechanical disruption from a mouse tumor. The suspension is diluted to $2x10^5$ cells in 3 mL in 0.3% agarose in Dulbecco's Minimal Essential Media (DMEM) plus 10% heat-inactivated Bovine Calf Serum (BCS) and plated in 60 mm tissue culture plates. For all cell types, except normal CFU, the 60 mm plates are first prepared with a hard agar bottom layer (3 ml of 0.6% agar in RPMI-1640 plus 15% BCS). The soft agar top layers are poured into the plates and allowed to solidify. The human cancer cell lines (obtained from American Type Tissue Collection, Manassus, VA), are maintained in cell culture. Cells are removed from their culture by a trypsin-collagenase-DNAase cocktail and treated similarly to the Colon 38 cells. A 3 mL suspension containing 30,000 cells in 0.3% agarose in DMEM plus 10% heat-inactivated BCS is plated in 60 mm tissue culture plates. For the normal cell type, CFU-GM, the femoral marrow of C57Bl/6 mice is flushed with Minimal Essential Media-alpha (MEM-alpha); 2 mL per femur. The cells are passed through an 18-gauge needle twice and the monodispersed suspension counted. A total of $1.5x10^6$ cells are plated in 3 mL of 0.3% agar with the

addition of 10% L-cell conditioned media, which provides colony stimulating factor, in MEM-alpha plus 10% BCS.

Zone assay methodology**:** A volume of 15 uL of each sample is dropped onto a 6.5 mm disks (Baxter filter disks). The disks are allowed to dry overnight and then placed close to the edge of the 60 mm tissue culture plates described above. The plates are incubated for 7-10 days and examined by an inverted stereo-microscope (10X) for measurement of the zone of inhibition. The diameter of the filter disk, 6.5 mm, is arbitrarily taken as 200 units. A zone of less than 300 units is taken as the extract is of insufficient activity to be of further interest. A difference in zones between solid tumor cells and either normal or leukemia cells of 250 units or greater defines solid tumor selective compounds. If the test material is excessively toxic at the first concentration, we then retest a range of dilutions of the agents (at 1:4 decrements) against the same tumors. At some dilution, appropriate activity is invariably obtained.

The solid tumor types were chosen because of their high frequency and mortality in terms of cancer deaths. Furthermore, there are very few "chemotherapy-cures" of these tumor types once distant metastatic spread has occurred, indicating the need for improved chemotherapeutic anticancer agents. The murine L1210 and human CCRF-CEM, and their respective normal marrow CFU-GM counterparts, are the reference cells of the assay, serving as a basis to define samples that are differentially cytotoxic to solid tumor cells, thereby defining solid tumor selectivity.

IC_{50} Assay:

Drug concentration-cell number studies (IC_{50} assay) are carried out against HCT-116 human colon cancer cells. These cells are grown in 5 mL culture medium (RPMI-1640 + 15% FBS containing 1% penicillin-streptomycin, and 1% Glutamine) at 37^0C and 5% CO_2 at a starting concentration of $5x10^4$ cells/T25 flask. On day 3, cells are exposed to different concentrations of the compound. Flasks are incubated for a further 5 days in a 5% CO_2 incubator at 37^0C and the cells harvested with trypsin, washed once with HBSS, resuspended in an HBSS-trypan blue solution and both total and viable cells (trypan-blue excluding cells) counted using a hemocytometer. The results are normalized to an untreated control. The IC_{50} value for viable cells is determined using Excell.

Clonogenic assay.

The clonogenic studies are carried out against HCT-116 cells grown in 5 mL culture medium (RPMI-1640 + 15% FBS) at 37^0C and 5% CO_2 at a starting concentration of 10^5 cells/T25 flask. On day 3, cells are exposed to different concentrations of the drug. Flasks are incubated for 2h, 24h or 168h in a 5% CO_2 incubator at 37^0C and the cells harvested with trypsin, washed once with HBSS and resuspended in HBSS. Cells are prepared in culture media containing 1% penicillin-streptomycin, and $5x10^{-5}$ M β-mercaptoethanol. After thorough mixing, aliquots are plated in 60 mm tissue culture plates so as to yield either 100 or 1000 cells per plate. The plates are incubated in a 5% CO_2 incubator maintained at 37^0C for 7 days. Colonies containing >50 cells are scored using a Stereozoom microscope (Bausch & Lomb, Model BVB-125, Rochester, NY). The results are normalized to an untreated control. Plating efficiency for the untreated cells is over 90%. Repeat experiments are carried out to define the cell survival range between 10^0 and 10^{-3} survival. A value defined as ${}_tS_{10}$ is used to define the effectiveness of the treatment. This value defines the concentration required to achieve a clonogenic survival of 10% of control for the exposures durations "t" h.

HPLC Assay

Quantation of catechol was accomplished using a Waters Corp. (Milford, MA, USA) model 2996 photodiode Array Detector and a model 2690 Separation Module. A Waters Symmetry C8 (4.6 X 150 mm) column was used as the stationary phase. The mobile phase was composed of 10% methanol in water at 1 mL/min. Catechol was detected using the photodiode array at 280 nm using an injected volume of 5 µL. Standard catechol sample concentrations were 0.0156, 0.03125, 0.0625, 0.125, 0.25 and 0.5 mg/mL. Cantron® was diluted 1:1000 prior to injection. The data was analyzed using the Waters Empower 2 (Build 2154) software package.

RESULTS

Cantron® is a blackish/brown liquid with a density of 1.12 g/mL containing a black precipitate (36 mg per mL of liquid) that settles quickly after shaking. The density of Cantron® after removing the precipitate was 1.1 g/mL. The typical dosing schedule for Cantron® is 1.5 mL, every 4 hours orally, even though Cantron® tastes particularly awful with a lingering metallic aftertaste. Lyophilization of samples of Cantron® yielded a value of 220 mg/mL for solid residue, a value which we used for subsequent biological studies.

The first decision point in our drug discovery paradigm is selectivity in the disk diffusion assay. This assay provides an initial assessment of the potential anticancer activity of a test sample. The results for a number of samples of Cantron® are presented in Table 1. The first sample tested (Cantron-original; batch prepared in 2010) demonstrated zone differentials between Colon38 and both L1210 leukemia and normal CFU-GM cells of greater than 250 units (bolded). Three further batches (prepared within the previous 5 years) provided by MRP demonstrated nearly identical results. A further MRP batch prepared over 5 years previous to our testing as well as a batch of Entelev® prepared by MRP in 1982 demonstrated identical results. A sample of the precipitate (removed by centrifugation and resuspended in water) demonstrated no cytotoxic activity.

Using our paradigm, a drug is moved forward if it demonstrates solid tumor selectivity in either test; in

Cantron's® case the selectivity for C38 against both the leukemia (L1210) and normal cell (CFU-GM) was sufficient to move Cantron® through the paradigm for further development.

The disk zone assay results for all of the known constituents of Cantron® are presented in Table 2. It is clear that catechol is the active, solid tumor selective constituent of Cantron®. Catechol, at 7 μg/disk produced a zone of 600 for C38 and a zone of 200 and 300 for L1210 and CFU, respectively.

THQ demonstrated some cytotoxicity against the cell lines tested but at a concentration over 60-fold greater than that found for catechol and without any solid tumor selectivity. The other compounds tested, Rhodizonic acid, Croconic acid, Triquinoyl, Inositol and Sodium Sulfate had little or no cytotoxic activity against any of the cell lines studied even at very high concentrations.

IC50 Assay

Because Cantron® and catechol demonstrated solid tumor selectivity and THQ demonstrated some cyctotoxicity, we next determined the IC_{50} values for Cantron®, catechol and THQ. Although outside of our normal paradigm we determined the IC_{50} of Cantron using 14 different cell lines, because it has been suggested that Cantron® is effective against many different cancer types (anecdotal and testimonial data described in the Introduction). We carried out the studies against 11 different solid tumor cell lines as well as 3 leukemia

TABLE 1. Disk Diffusion Assay Data for Cantron

	Conc. (μg/disk)	L1210	C38	CFU	H116	H125	CEM
Cantron-original	206	**400**	>1000	**250**	250	100	500
	52	**0**	**350**	**0**			
Cantron-251197	206	**450**	**800**	600	400	400	500
	52	**150**	**500**	**150**	50	50	
Cantron-62797	206	**500**	**850**	**500**	250	300	400
	52	**150**	**550**	400			
Cantron-32378	206	**450**	**700**	550	350	300	500
	52	**100**	**400**	**150**	50	0	
Cantron-5 years	206	**350**	**700**	500	400	400	250
	52	**150**	**450**	**100**			
Entelev-1982	206	**400**	**900**	**550**	500	400	350
	52	**250**	**550**	**150**			
Precipitate	546	0	0		0	0	

TABLE 2. Disk Diffusion Assay Data for Cantron Constituents

	Conc. (μg/disk)	L1210	C38	CFU-GM	H116	H125	CEM
Catechol	113	**750**	**>1000**				
	28	**450**	**>1000**	**550**	600	600	1000
	7	**200**	**700**	**300**			
Tetrahydroxyquinone	450	300	200	300	300	300	400
Rhodizonic acid	450	350	0	200	150		
Croconic acid	450	150	0	100	100		
Triquinoyl	450	150	100	100	100	50	
Inositol	235	0	0	0	0	0	
Sodium Sulfite	300	0	0	0	0		

TABLE 3. IC_{50} Values of Cantron®, Catechol and THQ Against a Panel of Human Tumor Cells. H116 - human colon cancer; Sar180 - murine sarcoma; H125M - human lung cancer; Panc-01 - human pancreatic cancer; OVC-5 - human ovarian cancer; U251N - human glioblastoma; CEM – human leukemia; MCF-7 - human breast cancer; LNCAP - human prostate cancer; L1210-murine leukemia; HEP-G2 – human liver cancer; MDA-235 – human breast cancer.

IC_{50} (μg/ml)								
	HCT-116	Sar180	H125	PANC-01	OVC-5	U251N	MCF-7	LNCAP
Cantron	20	11	30	10	23	23	25	32
Catechol	2.8	1.9	2.1	1.7	3.2	2.2	2.2	1.7
THQ	17	51	53	24	5.9	8.0	5.9	54
IC_{50} (μg/ml)								
	HEP-G2	Colon38	MDA-235	L1210	CEM			
Cantron		25	28	20	29			
Catechol	2.6	2.2	2.1	3.0	2.1			
THQ	8.5	19	5.8	49	7.0			

cell lines as indicated in Table 3. The IC_{50} values for Cantron® varied between 10 and 32 µg/mL with an average of 22.8 µg/mL. For catechol, the IC_{50} values varied between 1.7 and 3.2 µg/mL with an average of 2.3 µg/mL. It is clear that both Cantron® and catechol are toxic across many different cancer cell types. The ratio of the average values indicates that if all of the cytotoxicity of Cantron® were from catechol, it would represent about 10% of Cantron dry mass (about 22 mg/mL in Cantron). THQ cytotoxicity was much more variable, the IC_{50} values varied between from 5.9 to 54 µg/mL with an average of 23.7 µg/mL, a value 10-times greater than that found for catechol. Previous studies with V79 cells yielded an IC_{50} value for THQ of about 83 µg/mL (deSouza-Pinto et al., 1996). A 24 h exposure of THQ to HL-60 leukemia cells using an MTT assay yielded a range of 45 to 140 µM (7.7 to 24 µg/mL; MW of THQ=172) (Cavagis, Ferreira et al., 2006). The NCI tested Cancell® in their 60 cell line assay using a 48 h drug exposure and determined IC_{50} values in the 10-100 µg/ml range (http://dtp.nci.nih.gov/dtpstandard/cancerscreeningdata/index.jsp (Key words: Cancell, NSC 637907).

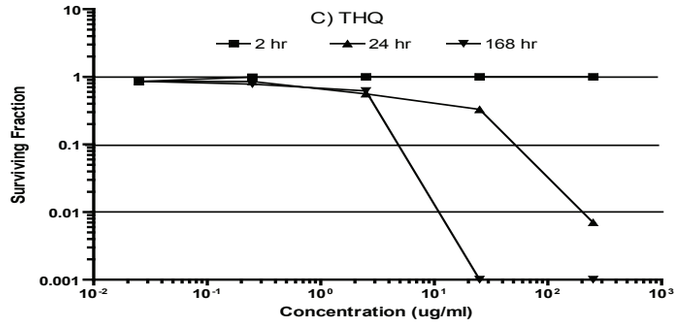
Clonogenic Assay

The next *in vitro* step in our development paradigm is to define a concentration-clonogenic cell survival assessment using HCT-116 cells. The clonogenic data is plotted in Figure 2 for Cantron®, catechol and THQ. The data yield $_2S_{10}$, $_{24}S_{10}$ and $_{168}S_{10}$ values for Cantron® of >2,200 µg/mL, 300 µg/mL and 5 µg/mL, respectively. For catechol, these values are 1500 µg/mL, 110 µg/mL, and 1.6 µg/mL. The catechol/Cantron® ratios for the latter 2 values are 0.37 and 0.32, indicating that if all of the cytotoxicity were from catechol, it would represent 35% of the composition of Cantron®. The $_tS_{10}$ values for THQ are >250 µg/mL, 50 µg/mL and 5 µg/mL, respectively for 2, 24 and 168 h. The $_{168}S_{10}$ value for THQ indicates that if all of the cytotoxic activity of Cantron® were attributed to THQ it would have to represent 100% of Cantron® by dry weight.

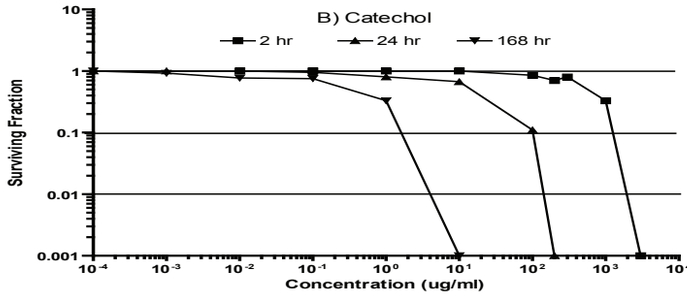
Quantitation of Catechol in Cantron®:

The most active ingredient in Cantron® according to zone assay, IC_{50} results and clonogenic data is catechol. HPLC quantitation of catechol in Cantron® yielded a concentration of 27 mg/mL. A further 18 samples of Cantron® from separate batch preparations (provided by MRP) were assayed and found to contain 28 (±1.9 s.d.) mg/ml catechol. Since Cantron® contains 220 mg/mL dry weight, catechol represents 12.7% of Cantron®. THQ was not detected in Cantron®. This is likely due to oxidation of THQ to RA which is further oxidized to UV invisible species (deSouza-Pinto, Vercesi et al., 1996; Hoffmann, Ciampi et al, 1987).

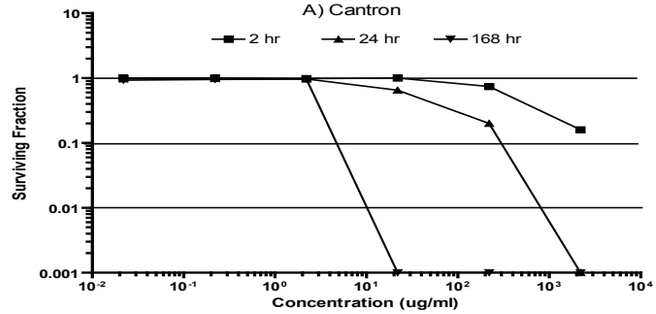
FIGURE 2. Clonogenic survival of HCT-116 cells exposed to different concentrations of A) Cantron, B) Catechol and C) THQ for either 2 h, 24 h or 168 hr.



X Title	2 hr		24 hr		168 hr
	Y	Ð	Y	Ð	Y
0.00250	1.000		0.800		0.770
0.02500	0.850		0.860		0.860
0.25000	0.990		0.850		0.780
2.50000	1.000		0.560		0.620
25.00000	1.000		0.330		0.001
250.00000	1.000		0.007		0.001



X Title	2 hr		24 hr		168 hr
	Y	Ð	Y	Ð	Y
0.00010			1.000		1.000
0.00100			1.000		0.920
0.01000	1.000		1.000		0.770
0.10000	1.000		0.950		0.750
1.00000	1.000		0.800		0.330
10.00000	1.000		0.670		0.001
100.00000	0.850		0.110		
200.00000	0.700		0.001		
300.00000	0.800				
1000.00000	0.330				
3000.00000	0.001				



X Title	2 hr		24 hr		168 hr
	Y	Ð	Y	Ð	Y
0.02200	1.000		0.970		0.930
0.22000	1.000		0.980		0.950
2.20000	0.980		0.970		0.970
22.00000	1.000		0.650		0.001
220.00000	0.740		0.200		0.001
2200.00000	0.160		0.001		0.001

DISCUSSION

Cantron® has been used for decades as an alternative therapy for the treatment of cancer and other serious human diseases (Anonymous, 1993). Upon examination by the National Cancer Institute it was determined to be ineffective as an anticancer agent (Anonymous, 1993; http://dtp.nci.nih.gov/dtpstandard/cancerscreeningdata/index.jsp - Key words: Cancell, NSC 637907). In our paradigm, however, it was found to be selectively toxic *in vitro* towards murine colon tumor cells (C38). This discovery moved Cantron® forward in our drug discovery program so that Cantron's® potential as an anticancer therapeutic agent could be further evaluated. The zone assay results also indicated that the only constituent of Cantron® that demonstrated a similar solid tumor selective profile was catechol. Approximately 7 µg/disk of catechol was comparable to 52 µg/disk of Cantron®, indicating that catechol might represent about 12.5% of the activity of Cantron® (identical to the 12.7% determined by HPLC assay of Cantron®).

The IC_{50} studies further indicated that the active ingredient in Cantron® was catechol. The ratio of the average IC_{50} values indicates that catechol accounts for 10% of the dry weight of Cantron® (again close to the 12.7% determined by HPLC assay of Cantron®). THQ was found to contribute some cytotoxic activity to the Canton®. It should be pointed out that a difference between our exposure duration (5 days) and that of NCI (2 days) is likely the basis for the significant difference in Canton® activity reported.

Finally, the clonogenic results indicated that although THQ contributed to the cytotoxic activity of Cantron®, the majority of the cytotoxicity was due to catechol. These studies also indicated that the most effective therapeutic schedule would likely be a chronic one given the high $_{2}S_{10}$ and $_{24}S_{10}$ values for Cantron® and catechol and the low µg/mL values for $_{168}S_{10}$. This prediction would seem to correspond to the dosing regiment (1.5 ml, six times a day) recommended by the manufacturers of Cantron®. From the above results, Cantron® has anticancer activity as well as anticancer therapeutic potential. Further study to determine any *in vivo* therapeutic effects in tumor bearing models need to be performed.

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