

A Flavonoid Fraction from Cranberry Extract Inhibits Proliferation of Human Tumor Cell Lines^{1,2}

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ABSTRACT In light of the continuing need for effective anticancer agents, and the association of fruit and vegetable consumption with reduced cancer risk, edible plants are increasingly being considered as sources of anticancer drugs. Cranberry presscake (the material remaining after squeezing juice from the berries), when fed to mice bearing human breast tumor MDA-MB-435 cells, was shown previously to decrease the growth and metastasis of tumors. Therefore, further studies were undertaken to isolate the components of cranberry that contributed to this anticancer activity, and determine the mechanisms by which they inhibited proliferation. Using standard chromatographic techniques, a warm-water extract of cranberry presscake was fractionated, and an acidified methanol eluate (Fraction 6, or Fr6) containing flavonoids demonstrated antiproliferative activity. The extract inhibited proliferation of 8 human tumor cell lines of multiple origins. The androgen-dependent prostate cell line LNCaP was the most sensitive of those tested (10 mg/L Fr6 inhibited its growth by 50%), and the estrogen-independent breast line MDA-MB-435 and the androgen-independent prostate line DU145 were the least sensitive (250 mg/L Fr6 inhibited their growth by 50%). Other human tumor lines originating from breast (MCF-7), skin (SK-MEL-5), colon (HT-29), lung (DMS114), and brain (U87) had intermediate sensitivity to Fr6. Using flow cytometric analyses of DNA distribution (cell cycle) and annexin V-positivity (apoptosis), Fr6 was shown in MDA-MB-435 cells to block cell cycle progression ($P < 0.05$) and induce cells to undergo apoptosis ($P < 0.05$) in a dose-dependent manner. Fr6 is potentially a source of a novel anticancer agent. *J. Nutr.* 134: 1529–1535, 2004.

KEY WORDS: • anticancer agent • flavonoids • cranberry • breast cancer

In spite of the many advances in cancer treatment, chemotherapy of solid tumors is still greatly limited by the lack of selectivity of anticancer drugs and by the recurrence of drug-resistant tumors. Finding a source of novel chemotherapeutics continues to be a focus of effort. Diets rich in grains, fruits, and vegetables are known to reduce cancer risk, implicating edible plants as potential sources of anticancer agents. A variety of compounds produced by edible plants have demonstrated anticancer activity (1–3); many of these belong to the flavonoid family (3–8). Berries, including cranberry (*Vaccinium macrocarpon* Ait. Ericaceae), are a rich source of many flavonoids. Extracts of cranberry were shown to inhibit the proliferation of tumor cell lines in vitro in a limited number of studies (9–11). Preliminary evidence from our group demonstrated that cran-

berry presscake (the material left after the juice is squeezed out of cranberries) was able to delay the growth and inhibit metastasis of human breast tumor MDA-MB-435 cells when provided as a diet supplement to tumor-bearing mice (12). However, cranberry has not been well characterized with respect to the mechanism by which it inhibits tumor cell proliferation.

The goal of this study was to characterize the components of cranberry presscake that were responsible for its antitumor activity. An extract of cranberry presscake was fractionated by standard chromatographic methods. An acidified methanol fraction, believed to contain flavonoids and proanthocyanidins (PACs),⁴ displayed antiproliferative activity against a number of different human tumor cell lines and was characterized for its capacity to arrest proliferation and induce apoptosis.

MATERIALS AND METHODS

Preparation of cranberry extracts. Cranberry extract and fractions were generously provided by Ocean Spray. The presscake is

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⁴ Abbreviations used: DMSO, dimethyl sulfoxide; EGCG, epigallocatechin gallate; Fr6, Fraction 6 of cranberry presscake extract; IC₅₀, concentration that inhibits proliferation by 50%; PAC, proanthocyanidin; PI, propidium iodide.

comprised of the remaining wet hulls after the juice is squeezed out. Presscake (3600 g) was blended in an equal weight of water (25°C), and filtered through sieves and a 1.5- μ m glass microfiber filter paper. After conditioning a reverse-phase C18 Flash 40M cartridge column (Biotage) with methanol and water, the entire sample was applied to the column. After eluting the permeate (Fraction 3), the column was washed with 250 mL of water (Fraction 4), 150 mL of 15% methanol:water (Fraction 5), and finally 325 mL of 1% acetic acid:methanol (Fraction 6, or Fr6). Each wash was collected and freeze-dried. A waxy material was obtained by eluting the column with 50% methanol and freeze-drying, and also from a precipitate obtained by cooling of Fr6. The various preparations, and some of their characteristics, are listed in **Table 1**. The concentrations used hereafter refer to those of the powder or residue from the freeze-dried fractions, on a weight:volume basis, upon solubilizing in the appropriate solvent.

In vitro assays of antiproliferative activity. Cell culture medium and fetal bovine serum were purchased from Invitrogen. Cell culture plastic ware was obtained from Invitrogen, Fisher Scientific, and VWR Canlab. Cultured cell lines were maintained in minimum essential medium α with nucleosides plus 10% fetal bovine serum and penicillin (50 kU/L)/streptomycin (50 mg/L) (growth medium). Cultures were incubated in a humidified atmosphere of 5% CO₂ at 37°C. Rapidly proliferating cells were utilized for establishing cultures of experimental cells, which were allowed to plate overnight in 96-well plates or 75-cm² flasks before manipulation. For cytotoxicity assays (96-well plates), exposure to cranberry preparations was initiated by the addition of 1 volume of growth medium (100 μ L) containing the extract at 2 times the desired final concentration, in triplicate wells for each condition. After 4 d of incubation, cell density was determined by viable staining using AlamarBlue (BioSource International), and a fluorescence-based plate reader (Wallac Oy) interfaced with a computer. The density of extract-treated cells was calculated as a percentage of that of control cells [cells treated with an equivalent concentration of dimethyl sulfoxide (DMSO), e.g., 0.1% for 100 mg/L]. To avoid any potential interference of cranberry constituents with the AlamarBlue assay, wells containing extract but no cells were included, and the values obtained were subtracted from those of cells exposed to the same conditions. The cranberry samples did not interfere significantly with the fluorescence of AlamarBlue.

The cell lines against which the cranberry preparations were tested were representative of a variety of human tumor types: MDA-MB-435 and MCF-7 (estrogen receptor-negative and -positive breast carcinomas, respectively), HT-29 (colon), DU145 and LNCaP (androgen receptor-negative and -positive prostate carcinomas, respectively), DMS114 (lung), SK-MEL-5 (melanoma), and U87 (glioma).

The MDA-MB-435 cell line was generously provided by Dr. Janet Price, University of Texas M. D. Anderson Cancer Center (Houston, TX). All other cell lines were purchased from American Type Culture Collection.

Flow cytometric assays

DNA (cell cycle) distribution. Fr6 treatment of MDA-MB-435 cells in 75-cm² flasks was initiated by the introduction of 0.2 volume of a 6-fold concentration of Fr6 in growth medium. At the indicated time points, cells were harvested by trypsinization, washed in PBS, and fixed using 70% ethanol. After staining of fixed cells with propidium iodide (PI) [0.02 g/L PI in PBS + 0.1% (v:v) Triton X-100 + 0.2 g/L deoxyribonuclease-free ribonuclease A; 20 min, 21°C], the cells were analyzed by flow cytometry (XL-MCL Flow Cytometer, Beckman Coulter). DNA (cell-cycle) distributions were analyzed using Multicycle for Windows Advanced DNA Cell Cycle Analysis software (Phoenix Flow Systems).

Apoptosis. Cultures were prepared and exposed to Fr6 as described above. Cells were harvested and, without fixing, were stained with fluorescein-conjugated annexin-V (BD Biosciences) and PI. Annexin-V binds to extracellular phosphatidylserine, a hallmark of apoptotic cells, but can also stain necrotic cells. PI can enter only cells in which the integrity of the membrane has been compromised, which can constitute necrotic or late apoptotic cells. Therefore, cells stained positively only for annexin-V are early apoptotic. Cells that were doubly positive for annexin V and PI were considered to be in late apoptosis.

Statistical analysis. The values for Fr6 that inhibited proliferation by 50% (IC₅₀) against the 8 cell lines were compared for differences using a nonparametric Kruskal-Wallis (χ^2) test, and a one-way ANOVA of log-transformed data, followed by Tukey's multiple comparison test. Differences in cell cycle distribution between treatments and controls, and among treatments, were compared using an unpaired, 2-tailed, Student's *t* test for groups with unequal variances. Where possible, these were confirmed for significance with a paired, 2-tailed *t* test. The induction of apoptosis was compared between Fr6 treatments and the DMSO-treated controls using a repeated-measures ANOVA followed by Dunnett's *t* tests. For comparison of apoptotic populations across d 1, 2, and 3 for a given treatment concentration, an unpaired *t* test was applied for groups with unequal variances because sample number varied among days. In all analyses, differences were considered to be significant if the *P*-value was <0.05.

TABLE 1

Properties of fractions of cranberry presscake extract

Fraction	Preparation	Expected constituents	Maximum stock concentration and solvent	Maximum concentration tested on cell lines	
			g/100 g	g/L	mg/L
1	Tomah hulls/freeze dried	Total presscake	100	10 in water (slurry)	500
2	Tomah hulls/freeze dried/extracted	Presscake insolubles	84	10 in water (slurry)	500
3	C18 first water filtrate/freeze dried	Glycosides, acids, soluble phenolics (proteins?)	5.55	10 in water	500
4	C18 water wash/freeze dried		0.5	10 in water	500
5	C18 15% methanol fraction/freeze dried	Sugars, organic acids	0.278	5 in 50% DMSO	100
6	C18 acidified methanol fraction/freeze dried	Flavonols, anthocyanins, flavan-3-ols, PACs	0.521	100 in DMSO	500
7	C18 wax from chilled methanol fraction/filtered/dry		0.07	5 in DMSO	50
8	C18 wax from 50% alcohol wash/freeze dried		0.02	3 in DMSO	30
9	C18 filtrate from wax alcohol wash/freeze dried		0.01	10 in DMSO	71

RESULTS

On the basis of the preliminary findings that cranberry presscake inhibited the growth of MDA-MB-435 breast tumors in animals (12), studies were begun to characterize the components of the presscake that were responsible for the antitumor activity. A warm-water extraction of presscake yielded a heterogeneous solution of proteins, sugars, phenolics, anthocyanins, and flavonoids and their derivatives. These different groups were separated using column chromatographic techniques (Materials and Methods; Table 1).

The various fractions were tested against a panel of cell lines representative of tumors from a variety of tissue types, using the maximum concentration of fraction that could be achieved based on the solubility of the material (Table 1), and with minimal toxicity due to the solvent. The lung tumor line DMS114 was inhibited by Fractions 1–4 and Fraction 9, by ~40–60% at the concentrations indicated. None of the other tested lines were growth inhibited by these fractions (data not shown). The only fraction that displayed consistent antiproliferative activity against all cell lines was Fr6. It was possible that this toxicity was due to a major change in the acidity of the medium, given the inherent acidity of cranberry. However, the pH of a 500 mg/L solution of Fr6 in growth medium was 7.44, compared with 7.65 for growth medium without additions. Therefore, the cytotoxicity was due directly to a chemical component of this fraction. Fr6 was further assessed by testing a range of concentrations against each cell line (representative experiment in Fig. 1). From these assays, the IC₅₀ concentrations of Fr6 were determined (Table 2). The prostate line LNCaP and the lung line DMS114 were much more sensitive to this preparation than the other cell lines.

The potential for Fr6 to kill cells as opposed to causing a transient arrest was first tested by determining the ability of cells to resume normal proliferation after inhibition by treatment with Fr6. Cell density was measured after a 4-d exposure to Fr6, and then again after a further 4 d in growth medium free of Fr6. In a preliminary experiment, cells exposed for 4 d to 300 mg/L of Fr6, which inhibited proliferation by ~80%, tended to proliferate much less slowly than untreated cells over the next 4 d without Fr6. This was further investigated

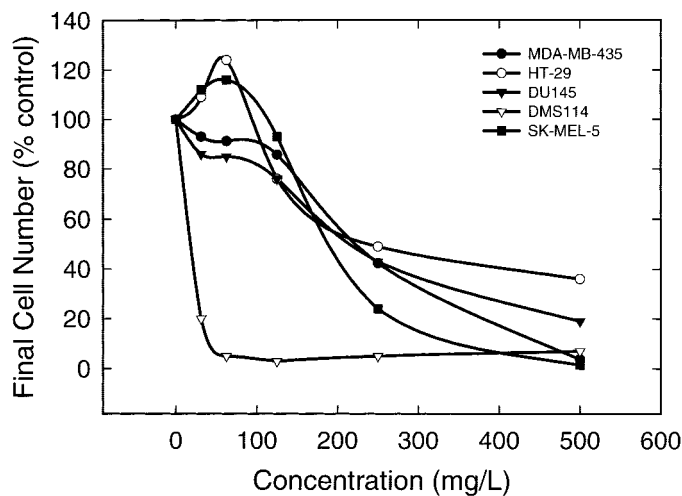


FIGURE 1 Inhibition of proliferation of human cancer cell lines by Fr6 over 4 d. The relative cell density (by vital fluorescent stain) was plotted as a percentage of vehicle-treated control. Single experiment, representative of a series summarized in Table 2 (with statistical analyses).

TABLE 2

Antiproliferative activity of Fr6 against a panel of tumor cell lines¹

Cell line	n	IC ₅₀ mg/L
MDA-MB-435	4	212 ± 50ab
MCF-7	3	147 ± 22cd
HT-29	4	168 ± 69ef
DU145	4	234 ± 75ghi
LNCaP	3	9.9 ± 4.2bdeijk
SK-MEL-5	4	147 ± 47jl
U87	3	77 ± 21agk
DMS114	4	21.1 ± 1.9acfh

¹ Values are means ± SD. Means with a common letter differ, *P* < 0.05.

over a range of concentrations (Table 3). The values at d 8 indicate the relative sizes of the populations (% of control) at this time point, and are not a measure of the change in cell number between d 4 and 8. However, these values are informative of the permanent effect that Fr6 can have on proliferative capacity. If proliferation was inhibited by >30% over the first 4 d, then the relative cell density (% of control) was significantly less by d 8 compared with d 4 (*P* < 0.05). This meant that the proliferation rate over the latter 4 d was less than that of control cells, and indicated a lasting effect of Fr6 on the health of the cells.

Flow cytometric analysis of DNA content was conducted on MDA-MB-435 cells treated with concentrations of Fr6 that had measurable effects on proliferation, to varying degrees, from minimal to almost complete inhibition (example treatment, Fig. 2). The percentage of cells in each phase of the cell cycle was calculated using MultiCycle software, and the results of 3 separate experiments were summarized (Fig. 3). With increasing concentration, cells accumulated increasingly in G2/M (dose-dependence *P* < 0.05), with a commensurate decrease in S-phase content (*P* < 0.05), but no change in G1.

As a more rigorous assessment of cell death induction, Fr6-treated cells were double-labeled with annexin V and PI. The total apoptotic population of cells (early plus late) is

TABLE 3

Effect of a 4-d exposure to Fr6 on the proliferation of MDA-MB-435 cells over the successive 4 d^{1,2}

Fr6 mg/L	d 4	d 8 (4 d without extract)
	% of control	
0	100	100
200	68.6 ± 4.8	47.6 ± 2.8
225	58.7 ± 5.3	36.2 ± 7.4
250	58.7 ± 3.5	28.9 ± 5.5
275	42.8 ± 14.2	17.3 ± 6.5
300	36.0 ± 1.5	17.9 ± 0.9
350	14.4 ± 2.9	3.3 ± 1.1

¹ Cell density was normalized to the respective control for that day; all values are means of determinations of fluorescence intensity in triplicate wells ± SD.

² At each Fr6 concentration, d 8 differs from d 4, *P* < 0.05.

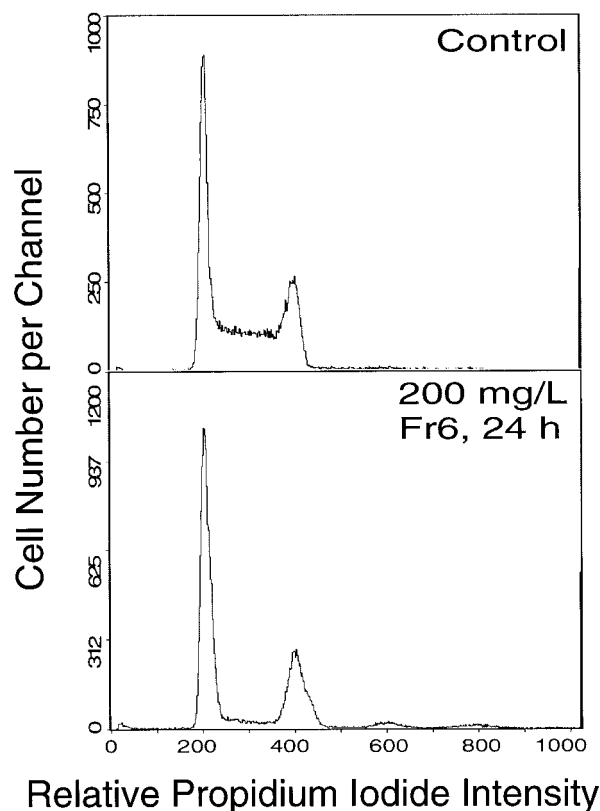


FIGURE 2 DNA analysis of Fr6-treated MDA-MB-435 cells. Rapidly proliferating MDA-MB-435 cells were exposed to 0 or 200 mg/L Fr6, and at 24 h tested for DNA content. Panels are representative of DNA histograms obtained and subsequently analyzed for DNA content (cell cycle distribution) (summarized in Fig. 3).

presented as a percentage of the entire population of cells (Fig. 4). This includes cells that had become detached from the surface of the flask as a result of poor health of the cells (including apoptotic and necrotic cells), but remained intact. (The flow cytometric analysis excluded debris and highly condensed cells by gating on intact cells, based on light scatter.) Cisplatin treatment was included as a known inducer of apoptosis ($n = 2$; therefore no P -value). The control cells underwent a small degree of apoptosis spontaneously. Fr6 treatment induced a significant degree of apoptosis within 1 d and continuing through d 3, leading to a significantly lower cell number (see values for relative proliferation in legend).

For 3 of these flow cytometric evaluations of apoptosis, cells were separated according to whether they had detached from the flask surface (floating cells) or remained attached, and then were distinguished as being in early apoptosis (annexin V-positive only) or late apoptosis (annexin V and PI double-positive) (only 2 values available for d 1 and 2) (Fig. 5). At 400 mg/L Fr6, at which proliferation was limited to <30%, the fraction of attached cells in apoptosis was increased at d 1 ($P < 0.05$). At both 300 and 400 mg/L, after d 1, the fraction of detached, apoptotic cells increased significantly day to day ($P < 0.05$). It is also possible that some of these detached cells were necrotic, but the fraction of detached, dying, and dead cells clearly increased greatly over that fraction at lower Fr6 concentrations and controls ($P < 0.05$).

In flow cytometric analyses of attached cells, fewer than 2% of particles were excluded by the light scatter-based gating system. Among nonattached cells, including those of controls,

up to 5–10% were sometimes excluded. The fraction of non-attached cells in a flask was generally <30% of the total population (and usually <10%), except that it was ~50% at 400 mg/L Fr6. Therefore, if anything, apoptosis may have been slightly underestimated at 300–400 mg/L, but not by >10%.

DISCUSSION

Particular families of plant constituents (phytochemicals) were shown to inhibit proliferation and enhance apoptosis in experimental cancer models (13–15). In a previous study, we found that cranberry presscake possessed anticancer activity against a human breast tumor line grown in immunodeficient mice (12). Therefore an extract of the presscake was fractionated to identify the family of compounds to which this anticancer activity could be attributed, and to determine the

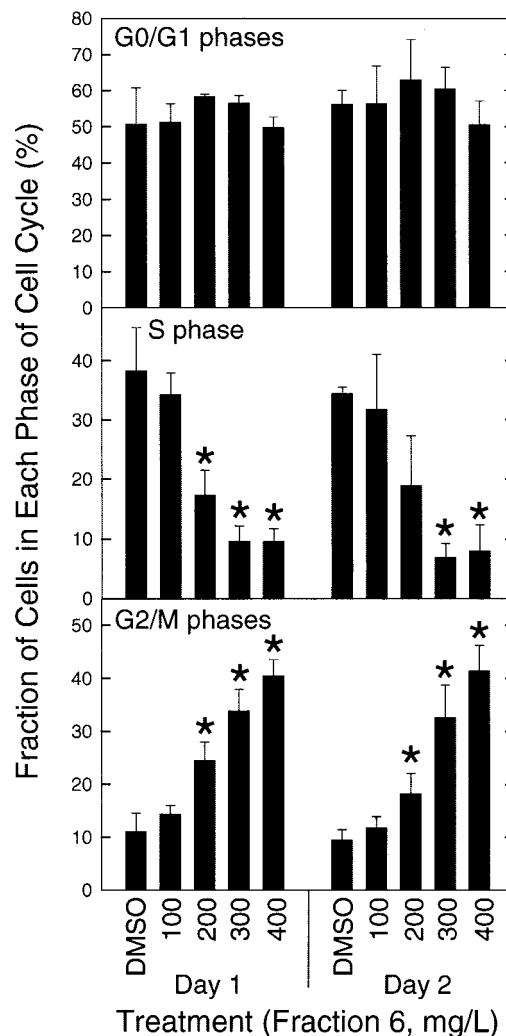


FIGURE 3 Cell cycle distribution analysis of Fr6-treated MDA-MB-435 cells. Rapidly proliferating MDA-MB-435 cells were exposed to 0 to 400 mg/L Fr6, and at 24 and 48 h were tested for DNA content (cell cycle distribution). The bar graphs are summarized data from 3 separate determinations (means \pm SD). The percentages of cells in G0/G1 phase, S phase, and G2/M phases are represented in their respective panels. *Different from the DMSO control, $P < 0.05$. For comparison to the antiproliferative effect of Fr6 on cells treated in these experiments, the relative proliferation measured after 3 or 4 d, for 100, 200, 300, and 400 mg/L Fr6, was (means \pm SD) 86.3 ± 7.1 ($n = 2$), 61.6 ± 6.0 (2), 36.2 ± 4.5 (2), and 20.9 ± 15.5 (3), respectively.

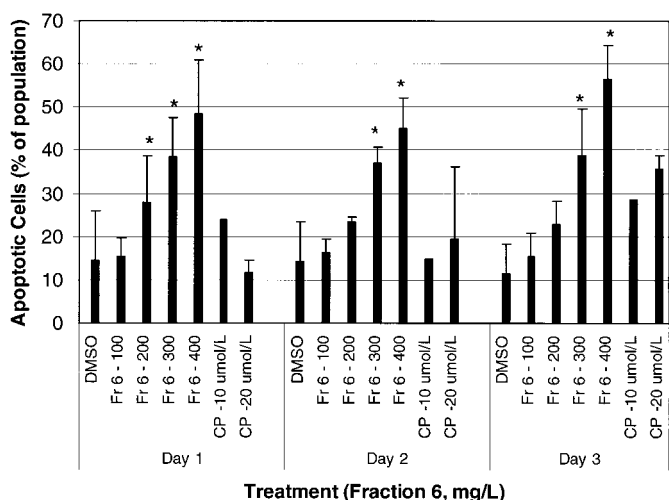


FIGURE 4 Apoptosis in Fr6-treated MDA-MB-435 cells. Rapidly proliferating MDA-MB-435 cells were exposed to 0–400 mg/L Fr6, and after 1, 2, and 3 d were tested for incidence of apoptosis. Bars indicate the percentage of the entire population of cells that was apoptotic, summarized from 3 to 4 experiments (means ± SD). *Different from the DMSO control, $P < 0.05$. Relative proliferation of treated cells, compared with DMSO-treated controls, after 4 d, for 100, 200, 300, and 400 mg/L Fr6, was (means ± SD) 86.7 ± 5.4 , 53.0 ± 10.3 , 28.4 ± 9.4 , and $17.6 \pm 5.5\%$, respectively ($n = 4$ for all).

mechanism by which these components inhibited proliferation. The antiproliferative activity of the presscake extract was associated with a fraction that eluted in acidified methanol (Fr6), and contained phenolics and flavonoids. Fr6 inhibited proliferation by arresting cell cycle progression, leading to apoptosis.

A better understanding of how cranberry phytochemicals inhibit tumor cell proliferation will enable them to be used more efficiently and perhaps synergistically with other antiproliferative agents. The panel of cell lines tested was selected to represent a spectrum of tumor types in which differences in sensitivity could suggest possible mechanistic information. Fr6 inhibited proliferation of all the cell lines in a dose-dependent manner, with IC_{50} values < 250 mg/L. This is a reasonable level of activity per dry weight of a heterogeneous preparation. Proliferation was completely inhibited by concentrations of ~400–500 mg/L. The slight enhancement of proliferation caused by low concentrations of Fr6 may be attributable to a hormetic effect, which is commonly observed in tumor cell lines treated with low concentrations of many different antiproliferative agents, including flavonoids (16). Because the breast tumor lines MCF-7 (estrogen-dependent) and MDA-MB-435 (estrogen-independent) exhibited similar sensitivity to Fr6, inhibition of proliferation was unlikely an antiestrogenic effect to any appreciable degree. DU145, HT-29, and SK-MEL-5 exhibited sensitivity similar to that seen in the breast tumor lines. U87, DMS114, and the androgen-dependent prostate line LNCaP exhibited progressively greater sensitivity to Fr6. The high sensitivity of LNCaP suggests that there may be a component of Fr6 that exhibits antiandrogenic activity.

To determine a broadly relevant mechanism responsible for the antiproliferative effect, the MDA-MB-435 cell line was chosen for further study. This cell line was the model tumor against which cranberry originally displayed antiproliferative activity in vivo (12). It was expected that the mechanism of antitumor activity against this cell line would be representa-

tive of that against the other cell lines because MDA-MB-435 is not estrogen- or androgen-dependent or -sensitive. Also, the high degree of sensitivity of lines such as LNCaP, U87, and DMS114 could be due to a characteristic specific to the individual lines. For the purpose of this study, a more general mechanism was sought that would apply to a range of cell lines from different tissue sources.

It was possible that Fr6 was preventing cells from proliferating without killing the cells. If so, we hypothesized that removal of Fr6 might result in resumption of proliferation. Exposure of MDA-MB-435 cells to Fr6 for 4 d and then culturing in the absence of Fr6 for a subsequent 4 d demonstrated that at concentrations of 200 mg/L and above, Fr6 had a permanent effect of inhibiting proliferation. Because cells must be stained and discarded after the fluorescence measurement in this assay, it was not possible to determine by how much the actual cell density changed between 4 and 8 d. It could only be determined that after 4 d free of Fr6, the treated population was a significantly smaller fraction of the control than at 4 d, indicating that proliferation proceeded much more slowly in treated cultures than in the controls. The very large differences in relative density between d 4 and 8 at ≥ 250 mg/L suggest that proliferation of these populations was negligible.

Studies were undertaken to determine whether Fr6 arrested cells in a specific phase of the cell cycle, or whether growth inhibition was due to a nonspecific, toxic effect on cells. This information can indicate whether Fr6 has a specific effect on cell biochemistry, lending a clue to its mechanism of action. The flow cytometric analyses indicated that treatment with components of Fr6 resulted in accumulation of cells in both G1 and G2/M, suggesting that several constituents of this preparation may exert different effects at different stages of the

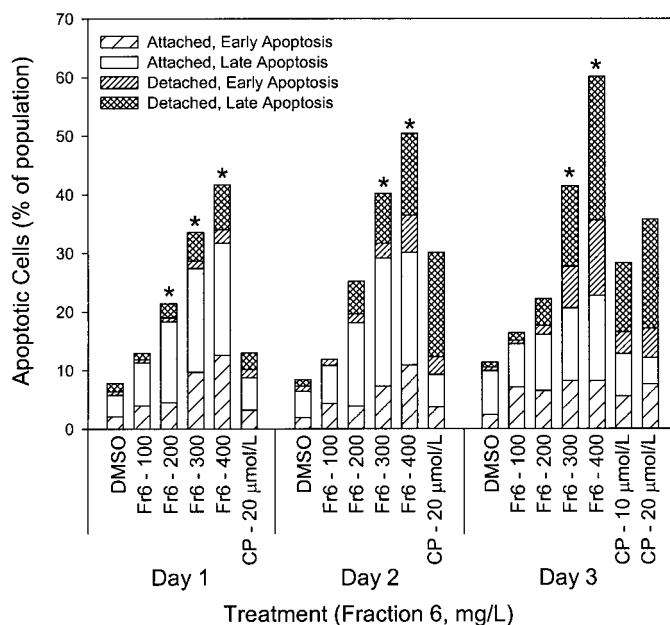


FIGURE 5 Apoptosis in Fr6-treated MDA-MB-435 cells, as a function of degree of staining and attachment to flask surface. Rapidly proliferating MDA-MB-435 cells were exposed to 0–400 mg/L Fr6, and after 1, 2, and 3 d were tested for incidence of apoptosis. Bars indicate the total percentage of cells in apoptosis, subdivided as detached or attached to the flask surface, in early or late apoptosis. (Error bars omitted for clarity; totals are included in Fig. 4.) Results are summarized from 2 or 3 experiments, depending on the concentration and time point. *Different from the DMSO control for total apoptotic population, $P < 0.05$.

cell cycle (14,17). The effects of Fr6 on cell cycle distribution were examined using concentrations that effectively inhibited proliferation. The percentage of cells in S-phase was significantly reduced by Fr6, in a concentration-dependent manner, after only 24 h. This could indicate that cells were blocked from exiting G1, although this normally would have led to an increase in the fraction of cells in G1. There was instead an accumulation of cells in G2/M, suggesting that cells were also blocked in this phase, and thus cells were not progressing from G2/M into G1. A putative arrest in both G1 and G2 phases is the simplest explanation for the observed decrease in S-phase cells, without a change in the G1 fraction (G1 arrest alone would increase G1 only; S or G2 arrest alone would decrease G1). Alternatively, if all cells undergoing apoptosis (Fig. 4) were in S-phase when they made this commitment, then this could account for the decrease in the S-phase fraction (observed, for example, in cells treated for 1 d with 300 mg/L Fr6; note the 25% increase in apoptosis with 25% decrease in S-phase fraction). There is no evidence at this point to indicate whether one or the other of these possibilities is correct, and perhaps both phenomena contribute to the observed alterations in cell cycle distribution.

Flavonoids commonly inhibit proliferation of tumor cell lines in the absence of cytotoxicity (18), which means that the cells could possibly resume proliferation if the treatment were removed. However, Fr6, at concentrations that induced cell cycle arrest, also induced apoptosis (Figs. 3, 4). Therefore, in vivo, a tumor treated with a sufficient dose of Fr6 would be expected to regress rather than sit dormant and then resume growth after treatment was stopped.

Specific phytochemicals that are components of cranberry inhibit tumor cell proliferation (4–8). However, there have been very few reports in which this activity was measured directly in cranberry extracts. Murphy et al. (15) recently identified 2 cranberry-derived phenolic compounds that inhibit proliferation of tumor cells in vitro. Otherwise, very little is known about the constituents of cranberry that are responsible for its anticancer activity, nor the mechanism by which they inhibit proliferation. An ethanol extract of cranberry reduced viability in 2 human tumor cell lines (9). A chloroform-methanol fraction from a crude acidified methanol extract of cranberry displayed antiproliferative activity against a panel of 7 tumor cell lines at concentrations from 16 to 250 mg/L (10). Against human hepatoma HepG2 cells, a cranberry extract inhibited proliferation with an IC_{50} of 14 g/L (11). The relatively low cytotoxicity of this acetone extract may have been due to the presence of inactive impurities such as sugars and proteins.

The most likely cranberry phytochemicals to have antiproliferative activity belong to the flavonoid family, which consists of the following 4 main groups: PACs, anthocyanins, flavan-3-ols (catechins), and flavonols. Cranberry flavonoids that are known to inhibit tumor cell growth and/or induce apoptosis include flavan-3-ols and the flavonols quercetin, myricetin, and resveratrol, although their contribution to these activities in cranberry extract, at the levels at which they are present, is not known. The flavan-3-ol with the best-established antiproliferative activity against tumor cell lines, both in vitro and in vivo, is epigallocatechin gallate (EGCG) (6,7,13,14). The actual EGCG content of cranberry is not known although the flavan-3-ol monomers (including catechin and epicatechin) constitute ~7% of total cranberry PACs (19). Catechin and epicatechin inhibit proliferation of prostate tumor cell lines (8). A large proportion of cranberry is comprised of 2- to 12-U polymers of these 3 flavan-3-ols

(20,21), but it has not been reported whether these polymers have antiproliferative activity.

Quercetin, myricetin, and resveratrol may also contribute to Fr6 antiproliferative activity (5,8,22,23). These flavonols inhibit the function of proteins involved in signal transduction (3,14,24) and gene transcription (25,26). They also exist in the form of glycoside conjugates, but it is not known to what extent these conjugates contribute to activity.

Other than the androgen-dependence of the LNCaP cell line, it is unclear what may contribute to the differences in the relative sensitivities of the 8 cell lines tested. The relative sensitivities do not reflect the status of p53 expression or integrity reported for these lines. MDA-MB-435 (27), DU145 (28), and HT-29 (29) express a mutant p53. MCF-7 (27), LNCaP (30), and U87 (31) have normal p53 expression. SK-MEL-5 is suspected, but not proven, to have mutant p53 (32). The p53 status of DMS114 is unknown. This pattern is not indicative of a significant contribution of p53 to the differences in cytotoxicity. However, there are a number of potential biochemical targets through which cell-cycle arrest and apoptosis may be induced. If Fr6 contains multiple constituents with more than 1 cellular target, it would be expected to exert multiple biological effects. Those effects could differ among tumor cell lines of different origins, depending on variation in the dependence of the cells on the affected pathways for growth and survival. The cyclin-dependent kinase inhibitors Waf1/Cip1/p21 and Kip1/p27 are differentially decreased or increased by treatment with different flavonoids at high or low concentrations, including quercetin, EGCG, silymarin, and genistein, resulting in cell death (apoptosis) or merely cell cycle arrest (5,14,17,33). Also, arrest may occur in G1 or G2, depending on the cell type (melanoma vs. breast carcinoma) (5,17). Therefore, in our study, the putative arrest of MDA-MB-435 cells in both G1 and G2/M upon treatment with Fr6 is potentially the effect of more than 1 component.

The results of this study collectively indicate that Fr6 contains components, possibly acting in concert, that inhibit the proliferation of the estrogen receptor-negative human breast tumor cell line MDA-MB-435 by blocking cell cycle progression and inducing apoptosis. The process used to prepare Fr6 and the other constituents of cranberry presscake extract can be undertaken on a preparatory scale, and is reproducible, as determined by analysis of a second batch of Fr6, prepared a year after the first, on 3 of the same cell lines (data not shown). Current studies to identify the components responsible for this cytotoxic activity could yield a novel anticancer agent.

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