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Biphasic Effect of Falcarinol on CaCo-2 Cell Proliferation, DNA Damage, and Apoptosis

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The polyacetylene falcarinol, isolated from carrots, has been shown to be protective against chemically induced colon cancer development in rats, but the mechanisms are not fully understood. In this study CaCo-2 cells were exposed to falcarinol (0.5−100 μM) and the effects on proliferation, DNA damage, and apoptosis investigated. Low-dose falcarinol exposure (0.5−10 μM) decreased expression of the apoptosis indicator caspase-3 concomitantly with decreased basal DNA strand breakage. Cell proliferation was increased (1−10 μM), whereas cellular attachment was unaffected by <10 μM falcarinol. At concentrations above 20 μM falcarinol, proliferation of CaCo-2 cells decreased and the number of cells expressing active caspase-3 increased simultaneously with increased cell detachment. Furthermore, DNA single-strand breakage was significantly increased at concentrations above 10 μM falcarinol. Thus, the effects of falcarinol on CaCo-2 cells appear to be biphasic, inducing pro-proliferative and apoptotic characteristics at low and high concentrations of falcarinol, respectively.

KEYWORDS: Polyacetylene; falcarinol; CaCo-2 cells; apoptosis; DNA damage; caspase-3; proliferation

INTRODUCTION

Several epidemiological studies have shown a negative correlation between a high intake of fruits and vegetables and the incidence of certain types of cancer (*1*, *2*). The protective effects of fruits and vegetables toward the development of cancer has primarily been ascribed to their content of vitamins, minerals, prebiotics (fibers), and groups of abundant non-nutritive antioxidants, such as carotenoids, phenolic acids, and flavonoids (*3*−*7*). It has also been proposed that such abundant single compounds or groups of compounds may be exploitable as dietary supplements for cancer prevention. However, studies on pure compounds in intervention studies have not always proved to be conclusive with respect to individual compound efficacy or mode of action. For example, epidemiological studies have shown a correlation between intake of vegetables rich in β-carotene and/or high levels of β-carotene in blood samples and a lower risk of cancer, whereas human intervention studies have shown that supplementation with β-carotene does not protect against development of this disease (*3*, *5*, *7*).

In most European countries and North America >50% of β-carotene intake is provided by carrots (*8*). A possible explanation for the observations described above is that carrots contain other bioactive secondary metabolites with a protective effect on cancer. This is a plausible explanation as carotenoids, phenolic acids, and flavonoids often co-occur in fruits and vegetables together with a number of other less abundant highly bioactive compounds.

Some of the less abundant bioactive compounds that are present in many Apiaceae vegetables are aliphatic C_{17}-polyacetylenes of the falcarinol type, of which some have been shown to exhibit anti-inflammatory characteristics (*9*, *10*), cytotoxic activities (*11*−*14*), and anti-platelet-aggregatory effects (*15*) as well as immune-stimulating effects (*16*). These secondary metabolites occur naturally in carrots and related vegetables such as celery, fennel, parsnip, and parsley of the Apiaceae plant family (*11*) and hence may contribute to the health-promoting effects of these vegetables including preventive effects on the development of cancer.

The most bioactive of the C_{17}-polyacetylenes occurring in carrots and related vegetables is falcarinol [(3*R*)-(9*Z*)-heptadeca-1,9-diene-4,6-diyn-3-ol; **Figure 1**], which is cytotoxic against various human tumor cell lines (*11*−*14*, *17*) and has a biphasic effect (hormesis) on cell proliferation of primary mammalian cells (*18*), the latter being characteristic for highly bioactive compounds (*19*). Furthermore, falcarinol is bioavailable in human subjects after ingestion of carrot juice, reported as persistent plasma concentrations of approximately 3 ng of falcarinol mL^{-1} (*19*), which is within the range in which the in

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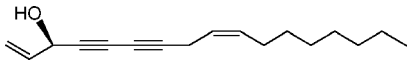

Figure 1. Chemical structure of falcarinol [(3*R*)-(9*Z*)-heptadeca-1,9-diene-4,6-diyn-3-ol] isolated from carrots and tested in the present study.

vitro data indicate that a positive physiological effect would be expected (*12*, *18*). In addition, falcarinol isolated from carrots has in a recent preclinical trial shown inhibitory effects on the development of azoxymethane (AOM)-induced colon preneoplastic lesions in rats, clearly indicating that falcarinol may have a protective effect toward the development of colon cancer and potentially other types of cancer (*20*). The mechanism for the inhibitory activity of falcarinol on cancer cells is still not known but may be related to its hydrophobicity and its ability to form an extremely stable carbocation, thereby acting as a highly reactive alkylating agent toward various biomolecules (*19*, *20*).

The aim of the present study was to investigate in vitro some of the possible mechanisms involved in the protective effect of falcarinol on the development of tumors and large aberrant crypt foci (ACF) demonstrated in vivo. A loss of apoptotic competence is a critical underlying feature of neoplastic progression. Indeed, the non-neoplastic colonic epithelium may also be unusually resistant to stress-induced apoptosis, thereby potentially increasing propensity for tumor development. Thus, the ability of dietary compounds to induce apoptosis may have a significant contribution to both the prevention and amelioration of colon cancer (*21*−*23*). In the present study we used the human colon carcinoma cell line CaCo-2 and focused on the effects of falcarinol on proliferation, DNA damage, and apoptosis.

MATERIALS AND METHODS

(−)-Falcarinol for Testing in Cell Cultures. (−)-Falcarinol [(3*R*)-(9*Z*)-heptadeca-1,9-diene-4,6-diyn-3-ol] (**Figure 1**) was isolated from carrot roots by column chromatography and preparative high-performance liquid chromatography (HPLC) according to the method described by Kidmose et al. (*24*). The purity of the isolated falcarinol was >98% as determined on an analytical Merck D-7000 Hitachi HPLC system equipped with a diode array detector operating between 200 and 600 nm. Falcarinol was monitored at 205 nm, and UV spectra were recorded between 200 and 450 nm. Chromatography was carried out on a reversed-phase Luna 3μ C18(2) 100A column (3 μm; 150 × 4.6 mm i.d., Phenomenex, Aschaffenburg, Germany) at 40 °C using the following linear programmed solvent gradient: CH_3-CN [HPLC grade (99.9%), Aldrich-Chemie, Steinheim, Germany]−H_2O [0−5 min (20:80), 10 min (50:50), 30 min (53:47), 45−50 min (65:35), 70−72 min (75:25), 90−95 min (95:5), 100−110 min (20:80)]. The water used for HPLC analysis was ultrapure generated by an Elgastat Maxima Analytica Water Purification System (Elga Ltd., Lane End, U.K.). All eluents for HPLC were filtered through a 0.45 μm Minisart SRP 25 filter (Bie & Berntsen, Rødovre, Denmark) and degassed with ultrasound for 20 min before use. The flow rate was 1 mL/min and the injection volume 20 μL. Retention time (Rt) and UV maxima for falcarinol were as follows: Rt = 68.3 min; λ_{max} 231, 244, 257 nm. (−)-Falcarinol was identified by mass spectrometry (EI, 70 eV), NMR (^{1}H and ^{13}C NMR and ^{1}H−^{1}H and ^{1}H−^{13}C correlation spectroscopy), and optical rotation as described previously (*20*, *24*), and the spectral data obtained were in accordance with literature values (*20*, *25*). Falcarinol was dissolved in DMSO, making a final concentration of 6.079 mM.

Cell Culture. CaCo-2 cells (European Collection of Cell Cultures, ECACC No. 86010202) were cultured at 37 °C in an atmosphere of 5% CO_2. The culture medium comprised DMEM (Sigma, Poole, Dorset, U.K.) supplemented with 10 mL/L nonessential amino acids (Sigma), 10 mL/L streptomycin and penicillin (Sigma), and 10% (v/v) fetal calf serum (Cambrex, Berkshire, U.K.). From stock culture, experiments were conducted within a range of a further 15 passages only. Cells were seeded in plates or flasks (Cell star, Greiner bio-one, Frickenhausen, Germany) and 24 h culture after they were exposed to falcarinol/DMSO (or DMSO alone in controls at a final concentration of maximum 1.7%) in fresh culture media and analyzed after 24−72 h as described for the individual assays.

Cell Proliferation and Adherence. Total cell number and the proportion of cells detached from the culture flasks were determined by microscopy using a hemeocytometer (Neubauer, Fischer, U.K.). Detached cells were recovered by centrifugation (1000*g*, room temperature) of aspirated culture medium, resuspended in PBS and counted. Adherent cells were harvested for counting using trypsin solution (Sigma). In addition, after 24 or 72 h of falcarinol treatment, 1 μCi of methyl [^{3}H]thymidine was added to each well for 4.5 h. Detached and adherent cells were harvested, pelleted by centrifugation, and resuspended in 150 μL of high-salt PBS solution (0.05 M Na_2HPO_4, 1 M NaCl, 0.002 M EDTA adjusted to pH 7.4). Samples were sonicated on ice for 2 × 10 s (Sonoplus 200, MS73 probe at 10%, Scientific Lab Supplies, Lanarkshire, U.K.), 3 mL of scintillation fluid (Ultima Gold, Packard, Berkshire, U.K.) was added, and disintegrations per minute (DPM) was counted in a β-counter (Packard 1900TR).

Active Caspase-3 Detection. CaCo-2 cultures were analyzed as the total cell complement, comprising both detached and adherent cells, or were split into adherent and floating cell fractions. The active form of caspase-3 was detected using a phycoerythrin (PE)-conjugated polyclonal active caspase-3 antibody (BD Pharmigen, BD Biosciences). Cells were permeabilized, fixed, and stained as recommended by the manufacturer and then analyzed by flow cytometry (FACS Calibur; Becton Divkinson, Oxford, U.K.). PE was excited at 488 nm and the fluorescent emission detected at 575 nm (FL-2 detector). A total of 10000 events were counted. Etoposide (10 μM), which induced a 20−25% increase in the number of cells expressing active caspase-3 (data not shown), was used to define regions of falcarinol-induced caspase activity using Cell Quest software (Becton Dickinson).

DNA Strand Breakage. After falcarinol exposure, CaCo-2 cells were harvested using trypsin. Cells were subsequently suspended in 85 μL of 1% (w/v) low melting point agarose in PBS, pH 7.4, at 37 °C, and applied on top of a precoated (1% w/v SMP agarose in PBS) frosted microscope slide. The agarose was allowed to set for 10 min at 4 °C, and slides were then incubated in lysis solution [2.5 M NaCl, 10 mM Tris, 100 mM Na_2EDTA, NaOH to pH 10.0, and 1% (v/v) Triton X-100] at 4 °C to remove cellular protein. This leaves the DNA as distinct nucleoids. Slides were aligned in a 260 mm wide horizontal electrophoresis tank containing buffer (1 mM Na_2EDTA and 0.3 M NaOH, pH 12.7) for 40 min before electrophoresis at 25 V for 30 min at 4 °C (the temperature of the running buffer was approximately 15 °C). Slides were washed 3 × 5 min at 4 °C in neutralizing buffer (0.4 M Tris-HCl, pH 7.5) and stained with 20 μL of 4′,6-diamidine-2-phenylindol dihydrochloride (DAPI) (1 μg/mL). DNA strand breakage was analyzed visually according to a scoring system where 100 images per gel (with replicate gels per flask) were assigned a value of either 0, 1, 2, 3, or 4 (from undamaged to maximally damaged) depending on the intensity of the fluorescence in the comet tail (*26*). This system has been extensively validated (R = 0.987) by image analysis (Komet 3.0, Kinetic Imaging Ltd., Liverpool, U.K.) as described previously (*27*). A positive control was included with each experiment using cells exposed to 200 μM H_2O_2 for 5 min on ice.

Statistical Analysis. Data are shown as means ± SEM, and statistical comparisons between experimental and control conditions were performed using unpaired two-tailed Student's t tests.

RESULTS

Cell Proliferation and Attachment. After 72 h, total CaCo-2 cell number increased significantly after exposure to low concentrations of falcarinol (0.5 and 1 μM) and decreased at higher concentrations (50 and 100 μM) (**Figure 2**). At concentrations ≥20 μM falcarinol exposure, the proportion of CaCo-2 cells detaching from the surface of the culture flask increased after 48 and 72 h (**Figure 3**).

Incorporation of [^{3}H]thymidine into CaCo-2 cells increased after exposure to 1−10 μM falcarinol for 24 and 72 h. At 20

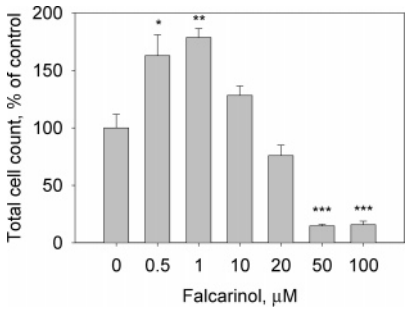

Figure 2. Total count of CaCo-2 cells after exposure to falcarinol for 72 h. The counts are expressed as percentage of counts in control treated cells (mean and SEM, $n = 4$). Values significantly different from control are indicated by *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

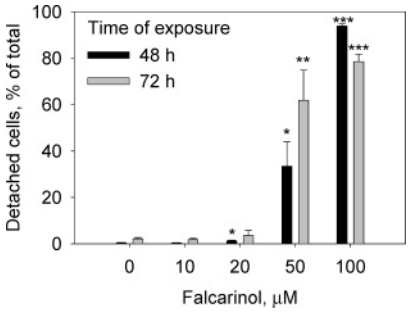

Figure 3. Detached CaCo-2 cells after exposure to falcarinol for 48 and 72 h. Detached cells are expressed as percentage of the total cell count (mean and SEM, $n = 4$). Values significantly different from control at each time point are indicated by *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

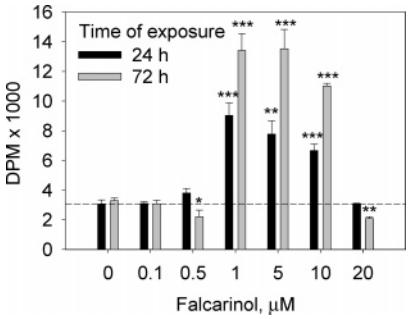

Figure 4. Incorporation of [^{3}H]thymidine into CaCo-2 cells after exposure to falcarinol for 24 h or 72 h. Results of 4.5 h of incorporation of [^{3}H]-thymidine are shown as disintegrations per minute (DPM) percentage of control (mean and SEM, $n = 4$). Values significantly different from control at each time point are indicated by *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

μM falcarinol exposure, [^{3}H]thymidine incorporation remained at control levels at 24 h and decreased at 72 h (**Figure 4**). Curiously, with 0.5 μM falcarinol, [^{3}H]thymidine incorporation also decreased after 72 h of exposure.

Caspase-3. Although some variation in background caspase activity was detected, the proportion of CaCo-2 cells expressing the active form of caspase-3 decreased significantly after 24 h of exposure to 10 μM falcarinol and after 72 h of exposure to 0.1 and 1 μM falcarinol (**Figure 5**). However, after 72 h of exposure to 100 μM falcarinol the expression of the active caspase-3 was significantly higher in CaCo-2 cells (**Figure 5**).

Analysis of adherent and detached cells within cultures treated with 50 μM falcarinol revealed that the proportion of cells expressing caspase activity was greater in detached as opposed to the attached cell population (**Figure 6**).

DNA Strand Breakage. DNA strand breakage was determined by single-cell gel electrophoresis (SCGE). Endogenous DNA strand breakage was significantly decreased in colon cells treated with 1 and 20 μM falcarinol for 24 h. Conversely, at higher concentrations, falcarinol significantly increased strand breakage in these cells (**Figure 7**). Low-dose falcarinol was similarly genoprotective after 72 h of exposure. At 72 h of exposure to 0.5 μM falcarinol, DNA strand breakage was significantly decreased compared with the control cells without falcarinol addition ($P < 0.05$), whereas at concentrations between 10 and 100 μM, falcarinol increased DNA damage dose dependently (**Figure 7**). As anticipated, DNA strand breakage was maximal in cells treated with 200 μM H_2O_2 for 5 min.

Among control cells without falcarinol exposure (**Figure 8A**) the DNA of the cells appeared to be intact and within the

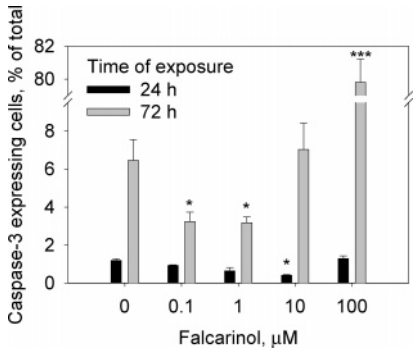

Figure 5. Percentage of cells expressing the caspase-3 active protein in CaCo-2 cells, after 24 or 72 h exposure to falcarinol (mean and SEM, $n = 4$). Values significantly different from control at each time point are indicated by *, $P \leq 0.05$; ***, $P < 0.001$.

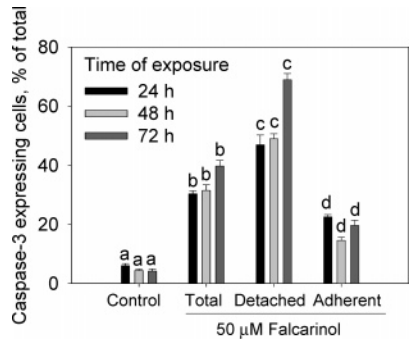

Figure 6. Percentage of cells expressing the caspase-3 active protein in CaCo-2 cells exposed to 50 μM falcarinol for 24, 48, and 72 h as total cells, adherent or detached cells (mean and SEM, $n = 3$). Significantly different values at each time point are indicated with different letters.

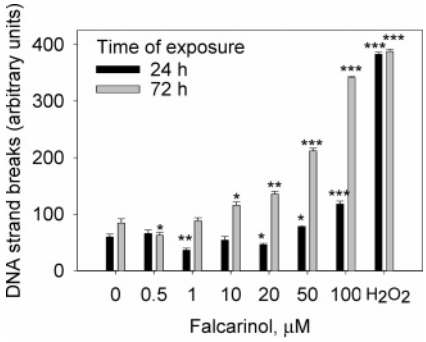

Figure 7. DNA strand breakage in CaCo-2 cells exposed to falcarinol for 24 or 72 h. Damage is expressed as arbitrary units (mean and SEM, $n = 4$). Values significantly different from control at each time point are indicated by *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

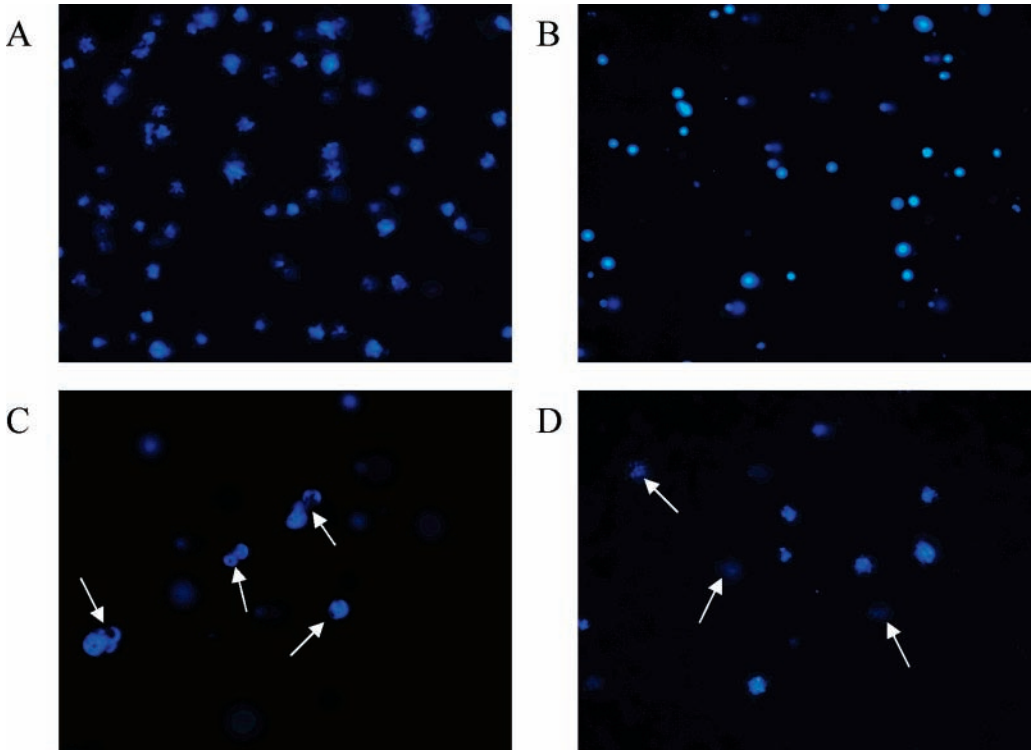

Figure 8. Fluorescent microscope images of DAPI-stained single-cell DNA after exposure to falcarinol and electrophoresis: (**A**) control cells (10×); (**B**) 10 μM falcarinol (10×); (**C**) 50 μM falcarinol (40×) (arrows indicate dark bodies in the cell nucleoids); (**D**) 100 μM falcarinol (10×) (arrows indicate "ghost" or remnant images described under Discussion).

boundaries of the nucleus. However, DNA damage, seen as increased fluorescence in the tail of the nucleoid, was observed in cells exposed to falcarinol at concentrations above 10 μM (**Figure 8B**). At falcarinol concentrations of 50 μM (**Figure 8C**) and 100 μM (**Figure 8D**) the fluorescent tail of the nucleoids had almost disappeared, indicating a very high degree of fragmentation. The DNA remaining in the nucleus appeared to be dense, and at higher magnification (40×) an increasing amount of dark bodies in the nuclear region was observed (**Figure 8C**).

DISCUSSION

In a rat model of colon carcinogenesis, in which colon preneoplastic lesions were induced by azoxymethane (AOM), the development of large aberrant crypt foci (ACF) and tumors was delayed or retarded when rats received freeze-dried carrots or pure falcarinol in the feed (*20*). Antiproliferative activity and in particular the induction of or sensitization to apoptosis may be a critical feature of dietary compound induced resistance to tumor development (*21*−*23*).

Studies of polyacetylenes in various cell cultures have shown reduced proliferation (*28*) and viability (*29*, *30*) as well as DNA fragmentation (*28*), and it has been suggested that the cytotoxicity of polyacetylenes (*12*, *14*) is due to inhibition of DNA replication (*31*) and induction of apoptosis. Induction of caspase-3 and caspase-9 activation together with up-regulation in proapoptotic Bax expression and down-regulation of the antiapoptotic Bcl-2 as markers of apoptosis has been shown in human SK-MEL-2 skin melanoma cells upon exposure to the polyacetylene dideoxypetrosynol A (*30*).

However, in this study, falcarinol exhibited opposing effects on the survival potential of the CaCo-2 cell line. At relatively low concentrations (0.5−10 μM) falcarinol exhibited a significant increase in cell number and/or tritiated thymidine incorporation. In addition, basal levels of DNA strand breakage and apoptosis-associated caspase-3 expression were decreased by <10 μM falcarinol exposure. Thus, although the relative contribution of and links between proliferation and decreased damage/stress are yet to be defined, a prosurvival effect appears to have been induced within CaCo-2 cells exposed to low falcarinol concentrations.

Conversely, DNA single-strand breakage and the apoptosis-associated features of caspase-3 activity and cell detachment, were significantly increased at higher falcarinol concentrations. The trigger for the antiproliferative and apoptotic effects has yet to be determined, but the relative timing and dose dependency of DNA damage relative to the expression of caspase-3 and cell detachment would implicate DNA damage as a target for further investigation.

Interestingly, several investigators have reported that alkaline SCGE can be used successfully to detect and discriminate between necrotic and apoptotic cell death (*32*−*35*). It has been proposed that in apoptotic cell death the nuclear DNA is extensively fragmented, allowing most of the low molecular weight DNA in the nucleoid to migrate into the agarose gel and to be lost during electrophoresis (*36*, *37*). Here, as revealed by SCGE, 50 or 100 μM falcarinol induced comet remnants or ghosts, whereby the DNA in the comet tail had virtually disappeared and only a "pin" of the comet head remained visible. This may reflect the significant nuclear degradation that occurs during apoptosis (*33*). It has been suggested that apoptotic cells can be detected earlier using SCGE than by conventional methods, as reviewed by Fairbairn (*34*), although this remains highly contentious (*36*, *38*).

Thus, falcarinol induces dose-dependent opposing effects on CaCo-2 proliferation and integrity. Paradoxical genotoxic/

genoprotective effects of phytochemicals are not uncommon, and several polyphenols display both toxic and protective effects in vitro and in vivo, as reviewed by Duthie et al. (*39*). For example, quercetin, a flavonoid, found in significant quantities in the human diet, increases the frequency of revertants in mutagenicity assays, induces chromosomal aberrations in animal cells in vitro, and increases DNA damage in human cells at concentrations above 100 μM (*26*), but is a potent cytoprotective agent against oxidative DNA damage at lower concentrations (*4*). A similar effect on proliferation as a function of falcarinol concentration has also been shown for mammary epithelial cells in vitro (*18*). However, this is the first report of apoptosis accompanying falcarinol antiproliferative activity and of apparently increased DNA integrity and suppressed background caspase activity accompanying the pro-proliferative effect of falcarinol.

Thus, falcarinol has the potential to modify cellular survival. Single-compound studies may highlight a therapeutic or dietary supplemental potential of a dietary compound. However, within the complexity of dietary intake, there will be a myriad of differing complementary and mutually opposing activities to which cells are exposed. In addition, as observed here, activities of single compounds vary dramatically with concentration or exposure longevity, and effects potentially vary as to the extent of stress, differentiation, and/or proliferative activity encountered at exposure (*40*, *41*).

Furthermore, we contend that it is the potential for activity to be expressed with a particular environment, for example, the developmental or pathological context in which exposure occurs, which will ultimately allow concepts of disease preventative benefit to be assigned. The colon epithelium represents a dynamic cellular environment, with apoptosis of differentiated cells occurring at the top of the crypt (*21*). However, in addition to an impairment of apoptotic competence during colon tumorigenesis, there may be a relatively high threshold for stress-induced apoptosis within the normal colon epithelium (*21*, *22*). It has been suggested that relative insensitivity to apoptosis induction may render the normal epithelium less susceptible to stress-induced apoptosis, allowing the accumulation of genomic damage (*21*−*23*). Thus, falcarinol-induced apoptosis may contribute an antitumorigenic activity within both these cellular environments.

The data presented here do not suggest an unambiguous mechanism through which falcarinol reduced AOM-induced ACF formation in rats. In the in vivo study the daily intake of falcarinol was approximately 150 μM (*20*), although this does not necessarily reflect the direct exposure concentration as bioavailability and metabolism of falcarinol in rats are presently unknown. It is therefore not possible at this time to conclude whether the exposure concentrations in the rat study corresponded to a low (<10 μM) or a high (>10 μM) exposure concentration of falcarinol. In this study it was revealed that the effects of falcarinol on CaCo-2 cells were biphasic, inducing pro-proliferative and apoptotic characteristics at low and high concentrations of falcarinol, respectively. Therefore, from the effects described here on CaCo-2 cells, as with a plethora of other dietary constituents proposed to contribute a determinant effect on the cancer preventative or therapeutic effect within fruit- and vegetable-rich diets, falcarinol cannot as yet be unambiguously described as a cytoprotective or cytotoxic agent.

ABBREVIATIONS USED

ACF, large aberrant crypt foci; AOM, azoxymethane; DAPI, 4′,6-diamidine-2-phenylindol dihydrochloride; DPM, disintegrations per minute; PE, phycerythrin; SCGE, single-cell gel electrophoresis; SMP, standard melting point agarose; LMP, low melting point agarose.

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