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Invited article

Chemopreventive characteristics of avocado fruit

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Abstract

Phytochemicals are recognized as playing an important role in cancer prevention by fruits and vegetables. The avocado is a widely grown and consumed fruit that is high in nutrients and low in calories, sodium, and fats. Studies have shown that phytochemicals extracted from the avocado fruit selectively induce cell cycle arrest, inhibit growth, and induce apoptosis in precancerous and cancer cell lines. Our recent studies indicate that phytochemicals extracted with chloroform from avocado fruits target multiple signaling pathways and increase intracellular reactive oxygen leading to apoptosis. This review summarizes the reported phytochemicals in avocado fruit and discusses their molecular mechanisms and targets. These studies suggest that individual and combinations of phytochemicals from the avocado fruit may offer an advantageous dietary strategy in cancer prevention.

Keywords: Avocado; Growth inhibition; Apoptosis; Reactive oxygen species; Cell signaling; Chemoprevention; Phytochemicals; Diet

Contents

1. Introduction

Cancer is one of the leading causes of death in the world. Its high incidence and mortality and lack of effective treatment have spurred extensive research on chemoprevention. It is generally accepted that the consumption of fruits and vegetables may reduce the risk of human cancers [1–3]. The protective effect of fruits and vegetables is thought to rely on multiple anticancer components. Efforts are continuing to identify individual and combinations of phytochemicals that selectively target precancerous and cancer cells. The avocado fruit is widely consumed as a food throughout the world, and this plant is also used for medicinal purposes. The health benefits of avocado may be due to its content of over 20 essential nutrients and various potentially cancer-preventing phytochemicals (Fig. 1). Additionally, avocados are low in calories, sodium and fat suggesting this fruit should be part of a healthy diet. While the health benefits of avocados have been known for many years, the cellular and molecular mechanisms of the phytochemicals responsible for cancer prevention are largely unknown. This review summarizes research at our institution and by other laboratories on

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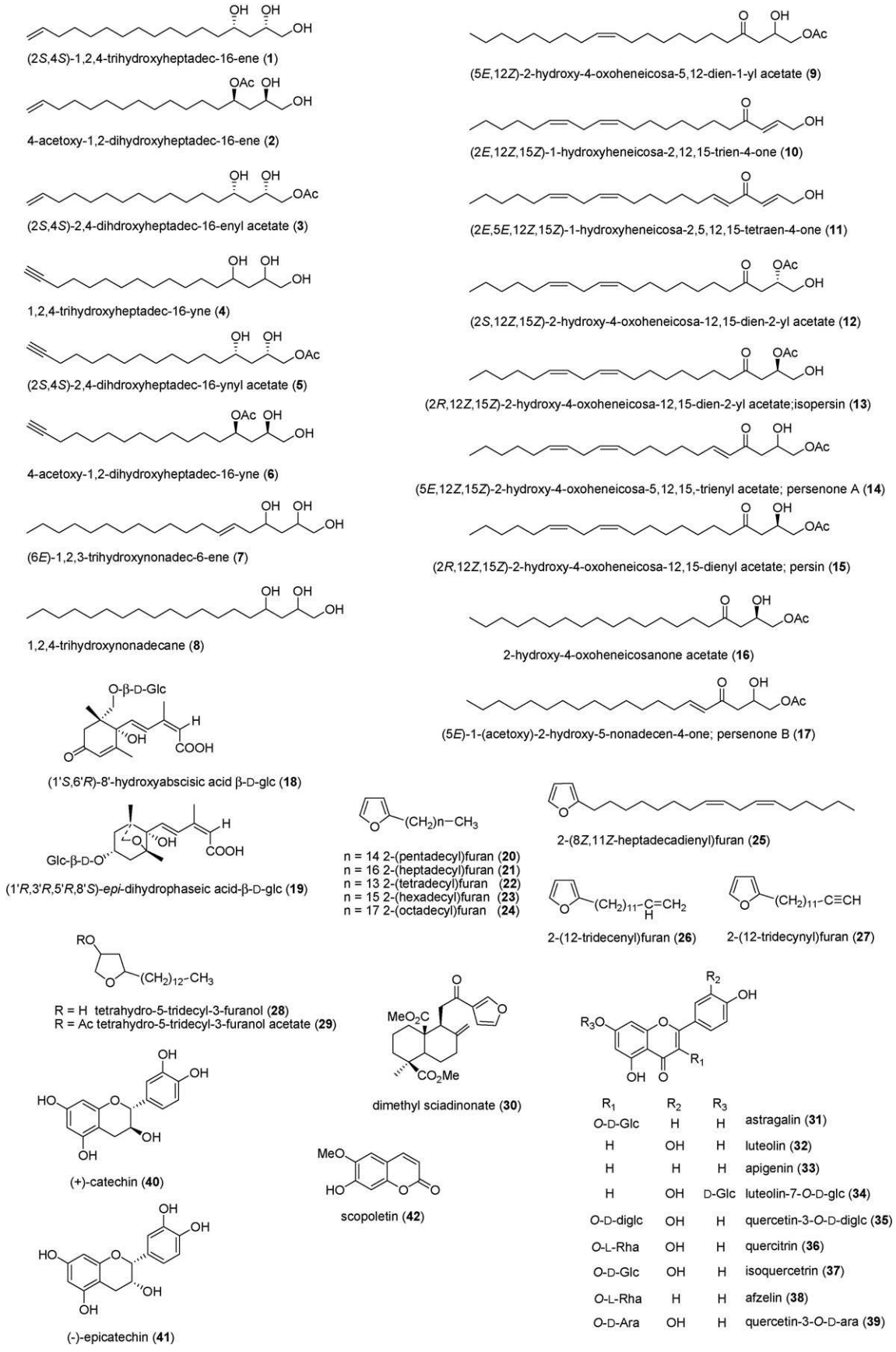

Fig. 1. Structures of secondary metabolite constituents of avocados.

the chemopreventive aspects of individual and combined phytochemicals isolated from avocado fruit in various cell culture and animal models.

2. Botany and ethnobotany of avocados

The avocado [*Persea americana* Mill.; syn. *P. gratissima* Gaertn., also referred to as *Laurus persea* L. (family Lauraceae)] is a New World species now widely cultivated around the world for its edible fruits, which are rich in volatile oil [4]. Altogether, about 70 species in the genus *Persea* occur in warmer regions of North, Central and South America, and 80 species in east and southeast Asia [5]. Other common names sometimes used for *P. americana* include "alligator pear", "avocato", and "ahuacate", and there are several varieties, including *drymifolia*, *floccosa*, *guatemalensis*, *nubigena*, and *steyermarkii* [4,5]. Avocado also has medicinal uses for wound healing and to stimulate hair growth (fruit pulp), as an aphrodiasiac and emmenagogue (fruits), and to treat dysentery and diarrhea [4]. Avocados are cultivated in the U.S. in California, Florida, and Hawaii and common varieties of use in commerce include "Bacon", "Fuerte", "Gwen", "Hass", "Lamb Hass", "Pinkerton", "Reed", and "Zutano" [6].

3. Biological activities of purified constituents of avocado

Examples of the major chemical (secondary metabolite) constituents of the various plant parts of avocado (*Persea americana* Mill.) (Lauraceae) reported to date are summarized in Fig. 1. These compound classes may be divided into alkanols (also sometimes termed "aliphatic acetogenins") (**1**–**17**), terpenoid glycosides (**18**, **19**), various furan ring-containing derivatives (**20**–**30**), flavonoids (**31**–**41**), and a coumarin (**42**). It is convenient to discuss the biological activities of these substances as each structural class is dealt with in turn.

The highly functionalized alkanols (**1**–**17**; Fig. 1) [7–10] of avocado have exhibited quite diverse biological activities thus far. For example, Oberlies et al. isolated 1,2,4-trihydroxyheptadec-16-ene (**1**), 1,2,4-trihydroxyheptadec-16-yne (**4**), and 1,2,4-trihydroxynonadecane (**8**) from the unripe fruits of *P. americana*, and found these substances to be moderately cytotoxic when evaluated against a small panel of cancer cell lines [8]. Kawagishi et al. isolated five alkanols from avocado fruits with "liver suppressing activity" (as determined by changes in plasma levels of alanine aminotransferase and aspartate aminotransferase), including compounds **9**–**11** [10]. An ethanol-soluble extract of the dried leaves of avocado exhibited anti-inflammatory activity in a carrageenan-induced edema protocol, with 1,2,4-trihydroxyheptadec-16-ene (**1**) being obtained from the active fraction [11]. In addition, 1,2,4-trihydroxyheptadec-16-ene (**1**), and related compounds **2**–**8**, purified from the seeds of *P. americana,* all exhibited moderate activity against epimastigotes and trypomastigotes [12].

Persin [**15**; (2*R*,12*Z*,15*Z*)-2-hydroxy-4-oxoheneicosa-12,15-dienyl acetate], a constituent of avocado leaves, is regarded as a toxin for lactating livestock [13]. In addition, persin has been found to reduce the larval growth of the beet armyworm, *Spodoptera exigua* [9], and is a known antifungal agent against *Colletotrichum gloeosporioides* [14]. Two analogs, persenones A (**14**) and B (**17**), along with persin (**15**), were found to inhibit superoxide (O_2^-) and nitric oxide (NO) generation in cell culture, and may thus serve as cancer chemopreventive agents in inflammation-related organs [14]. Persin (**15**) and three further analogs, compounds **3**, **5**, and **14**, showed inhibition of acetyl CoA carboxylase (ACC) activity, in the IC_{50} value range 4.0–9.4 μM [15]. Persin (**15**) has been noted to be labile under acid conditions, whereupon it produces a furan ring-containing analog [9]. The leaves of 17 avocado cultivars were investigated for their content of persin (**15**), and all but two of these contained discernible amounts of this compound (range 0.4–4.5 mg/g) [16].

The glucosylated abscisic acid derivatives (1′*S*,6′*R*)-8′-hydroxyabscisic acid β-D-glucoside (**18**) and (1′*R*,3′*R*,5′*R*,8′*S*)-*epi*-dihydrophaseic acid-β-D-glucoside (**19**; Fig. 1) were isolated from the seeds of *P. americana*, although no biological activities were attributed to these compounds [17].

Compounds **20**–**30** (Fig. 1) are furanoid constituents of avocados, and have been isolated and structurally characterized or chemically synthesized by several different groups [7,18–21]. These compounds have been termed "avocadofurans" and subjected to literature review, primarily from the point of view of the effects of structural modification on their resultant antibacterial, antifungal, and insecticidal activities [22].

Several flavonoids (**31**–**41**) (Fig. 1) have also been isolated from the leaves and seeds of avocados, with most of these being common flavones of wide distribution in the plant kingdom [23–26]. Some of these are biologically active, such as quercitrin (**36**), which showed virustatic effects by inhibiting HIV syncytium formation and viral p24 antigen formation [24]. An extractive of avocado leaves inhibited herpes simplex virus type I (HSV-1) and Aujeszky's disease virus and adenovirus type 3 (AD3). Bioactivity-guided fractionation led to the isolation of afzelin (**38**) and quercetin 3-*O*-D-arabinopyranoside (**39**), as inhibitors of acyclovir-resistant HSV-1 (20). The methanolic extract of avocado seeds showed antioxidant activity in an AMVN-induced methyl linoleate peroxidation assay, and catechin (**40**) and epicatechin (**41**) were isolated as major active components [26]. Also obtained as a constituent of avocado leaves is the commonly occurring plant coumarin, scopoletin (**42**, Fig. 1) [23].

There have been several reports of biological activity exhibited by extracts prepared from plant parts of avocados (*P. americana*) for which the active principles have not been characterized structurally, including anticonvulsant (in mice using standard convulsant drugs to cause seizures) [27], antioxidant (inhibition of NADPH oxidase activity in HL-60 cells and of epithelial xanthine oxidase in AS52 cells) [28], "chondroprotective" (i.e., reducing degenerative changes in granulomatous tissue) [29], rat skin lysyl oxidase inhibitory [30], and periodontal-disease related [including inhibition of matrix metalloproteinase (TIMP-1 and TIMP-2) secretion in human fibroblasts] [31] activities.

4. Inhibition of cell growth

Cellular proliferation is a carefully orchestrated process through which cells enter the cell cycle in G_1, duplicate their DNA in S, prepare for mitosis in G_2 and divide in mitosis. This complex process requires precise timing and coordination of many different types of proteins promoting [cyclins and cyclin dependent kinase (cdk)] and impeding (cdk inhibitors) the progression of the cell through the cell cycle [32,33]. Upstream oncogenes and tumor suppressor genes have been identified as positive and negative regulators of these cell cycle proteins. In most tumor cells, these genes regulating progression through the cell cycle are often mutated leading to the high levels of cellular proliferation [34–37]. As cellular proliferation is often many times greater in tumor cells than normal cells, toxic chemotherapeutic drugs often target DNA or proteins regulating cell cycle progression causing cells to arrest in the G_0/G_1, S, and G_2/M phases of the cell cycle [38,39]. For a chemopreventive agent to be effective in long-term use, it should exhibit minimal toxicity toward normal cells. Indeed, phytochemicals and extracts prepared from edible fruits have been identified with low toxicity while being selective inhibitors of tumor cell growth.

Extracts prepared from California Hass avocado fruit using a previously published plant extraction scheme [40,41] were tested for growth inhibition of the normal (TE1177), premalignant (SCC83-01-82) and malignant (SCC83-01-82CA) human oral cell lines [35]. Among the fractions tested, the chloroform extract (code: D003) was identified as one the more selective growth inhibitors of both the premalignant and malignant human oral epithelial cell lines (Fig. 2). The GI_{50} for the malignant cell line was 14 μg/ml, while that for the normal cell line was 2.7-fold higher at 38 μg/ml. Further fractionation of this extract, using a silica gel column chromatography with a gradient solvent system of increasing polarity, suggested that the active components are largely distributed in the less polar sub-fractions. This extract

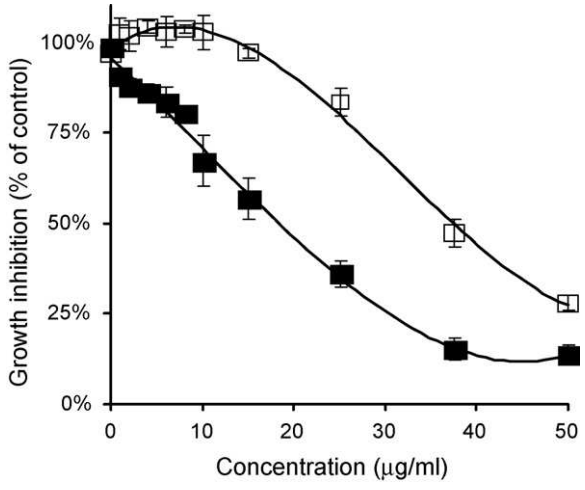

Fig. 2. A chloroform extract (D003) of avocado selectively inhibits the growth of the malignant and premalignant (not shown) human oral cell line (■). The normal human oral cell line (□) requires 2.7-fold higher concentration of extract for 50% growth inhibition. Cells were treated with extract for 3 days and relative cell number was determined using methylene blue.

(D003) decreased the levels of cyclin D, cyclin A, and cdk2, while increasing the levels of p21WAF1/Cip1. These data suggested that the phytochemicals in the chloroform extract (D003) inhibit growth by targeting cell cycle regulatory proteins.

Other studies have identified a number of chemicals found in various parts of the avocado as targeting the cell cycle. Persin (**15**, Fig. 1) induced G_2/M phase arrest in human breast cancer cell lines MCF-7 and T-47D cells, but did not significantly affect cell cycle distribution progression of the human breast cancer cell line MDA-MB-231. Consistent with the cell cycle change, persin reduced the levels of cyclin B1, cyclin A and D1 in MCF-7 and T-47 but not in the MDA-MB-231 cell line. Persin may also act as a microtubule stabilizer [42]. Another class of phytochemicals found in a wide number of fruits including avocado is glycosylated quercetin (**35–37**, and **39**, Fig. 1) and its analogs, luteolin (**32**, Fig. 1) and apigenin (**33**, Fig. 1) [43]. Quercitrin (**36**, Fig. 1) may be converted to quercetin by human intestinal bacteria or other enzymes [44,45]. Quercetin induces a G_2/M arrest in several cell types, including U937, lung cancer, prostatic carcinoma cells (PC-3) cell lines and normal tumor fibroblast cells [46] [47]. Similar to the effects of persin, G_2/M arrest may be caused by a substantial decrease in the expression of Cdc2, cyclin B1 and increase in p21 [47–49]. In fibroblast cell lines, G_2/M arrest did not occur in p53-knockout cells, suggesting G_2/M arrest in fibroblast cells is p53 dependent [50]. Whereas quercetin induces a G_1 arrest in primary and HPV-16 E6/E7 transformed human keratinocytes and human hepatoma cell line [51]. Quercetin down-regulated the expression of the Cdc6, CDK4 and cyclin D1 cell cycle genes, in concert with growth inhibition and cell cycle arrest in Caco-2 cells [52]. In vivo, quercetin modulated the expression and phosphorylation of cdc-2 and cyclin B1, and inhibited the Ki-67 index by 66.0% in prostate tumor xenograft SCID mice [53]. Quercetin reduced the steady state expression levels of Ras proteins in primary colorectal tumors and human epithelial cells [54,55].

5. Mechanisms of apoptosis induced by avocado phytochemicals

Apoptosis is an energy-requiring tightly regulated form of cell death involving multiple signaling pathways including cell surface death receptors and disruption of the mitochondria [56–64]. In the death receptor pathway, FADD/TRADD adaptor proteins recruit and activate the initiator procaspase 8 or 10, which then activate caspase 3, Bid and/or Bim. Cleavage of Bid results in changes in the mitochondrial membrane releasing cytochrome c and procaspase 9 to complex with Apaf-1 to form the apoptosome. The mitochondrial membrane can be disrupted by direct interaction with many different types of chemicals, or perturbing the balance of pro (Bid, Bax, Bak)- and anti (Bcl-2, Bcl_{xl})-apoptotic proteins.

Analyses of the chloroform extract (D003) by flow cytometry and Western blotting indicated a large portion of cells were apoptotic with the appearance of a large sub-G_1 peak and cleavage of PARP (Fig. 3). This was confirmed and the pathways involved in apoptosis were determined by analyzing the effects

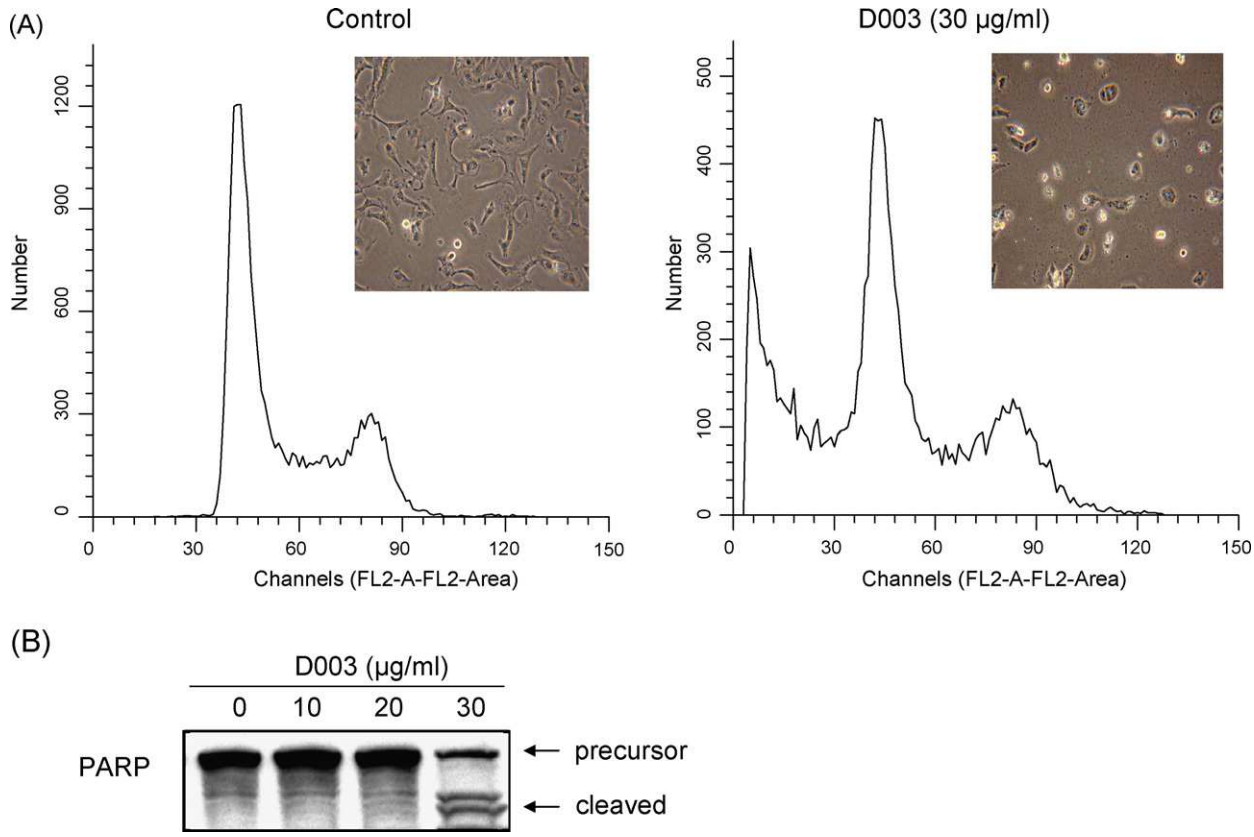

Fig. 3. The malignant cell line was treated with 30 μg/ml of the chloroform extract (D003) for 24 h. (A) The extract induced significant morphological change (inset) and a Sub-G_1 fraction of cells as analyzed by flow cytometry; (B) Western blot shows PARP cleavage in response to the treatment.

of the chloroform extract (D003) on the activation of caspases 3, 8, and 9. Activation of caspase 8 and 9 were observed as early as 6 h after the addition of the chloroform extract (D003), while activation of caspase 3 and cleavage of PARP appeared later within 12 h. To further define the sequence and role of caspase activity in the chloroform extract (D003) induced apoptosis, the CA cell line was treated with the chloroform extract (D003) in the presence of z-VAD-fmk, a pan-caspase inhibitor. As expected, co-incubation with z-VAD-fmk completely blocked the chloroform extract (D003) induced apoptosis, as indicated by the absence of caspase 3 activation and PAPR cleavage. To distinguish between the mitochondria/caspase 9 and death receptor/caspase 8 pathways in avocado extract induced apoptosis, cells were treated with D003 extract in the presence of z-IETD-fmk, a caspase 8 irreversible inhibitor. The caspase 8 specific inhibitor blocked the extract induced caspase 8 activation and PARP and caspase 3 cleavage, a downstream product of caspase 8 activation. To confirm this, a cell line with stable expression of dominant negative FADD, i.e., CA/GFP/FADD-DN, was established by transfection of the malignant cell line with FADD-DN and control GFP vector plasmids. The expression of FADD-DN partially attenuated the chloroform extract (D003) induced activation of caspase 8 and 3 and the cleavage of PARP (Fig. 4). This suggests that phytochemicals in the chloroform extract (D003) induce apoptosis via the death receptor, FADD, pathway. Further purification and characterization of the phytochemicals from this extract should identify specific phytochemicals targeting FADD and other molecular pathways. Studies using persin (**15**, Fig. 1) showed that apoptosis in human breast cancer cells was Bim dependent [42]. The Hs578.T and MDA-MB-231 cell lines with constructively low Bim expression are not sensitive. Bim expression silenced by siRNA in MCF7 is more resistant to apoptosis induced by persin. Quercetin- and luteolin-induced apoptosis appears to be associated with the down-regulation of bcl-2 and bcl_{xl} and up-regulation and bax [47–49,65]. Quercetin down-regulates the expression of bcl-2 in the xenograft B16M-F10 cells which facilitates endothelium-induced tumor cytotoxicity in B16M-F10 cells [66]. In vivo, quercetin suppresses aberrant crypt foci in an azoxymethane-induced rat colon cancer model via the mitochondrial pathway due to an increase in the Bax/Bcl-2 ratio [67].

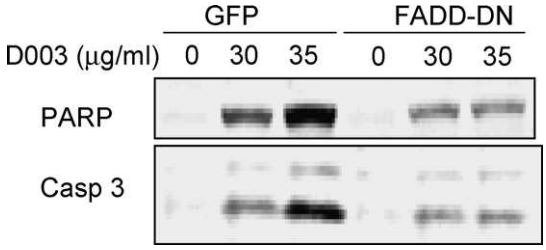

Fig. 4. A malignant oral cell line with stable expression of dominant negative FADD (FADD-DN) and GFP was treated with D003 for 8 h. Western blot analyses indicate the levels of PARP and Caspase 3 protein.

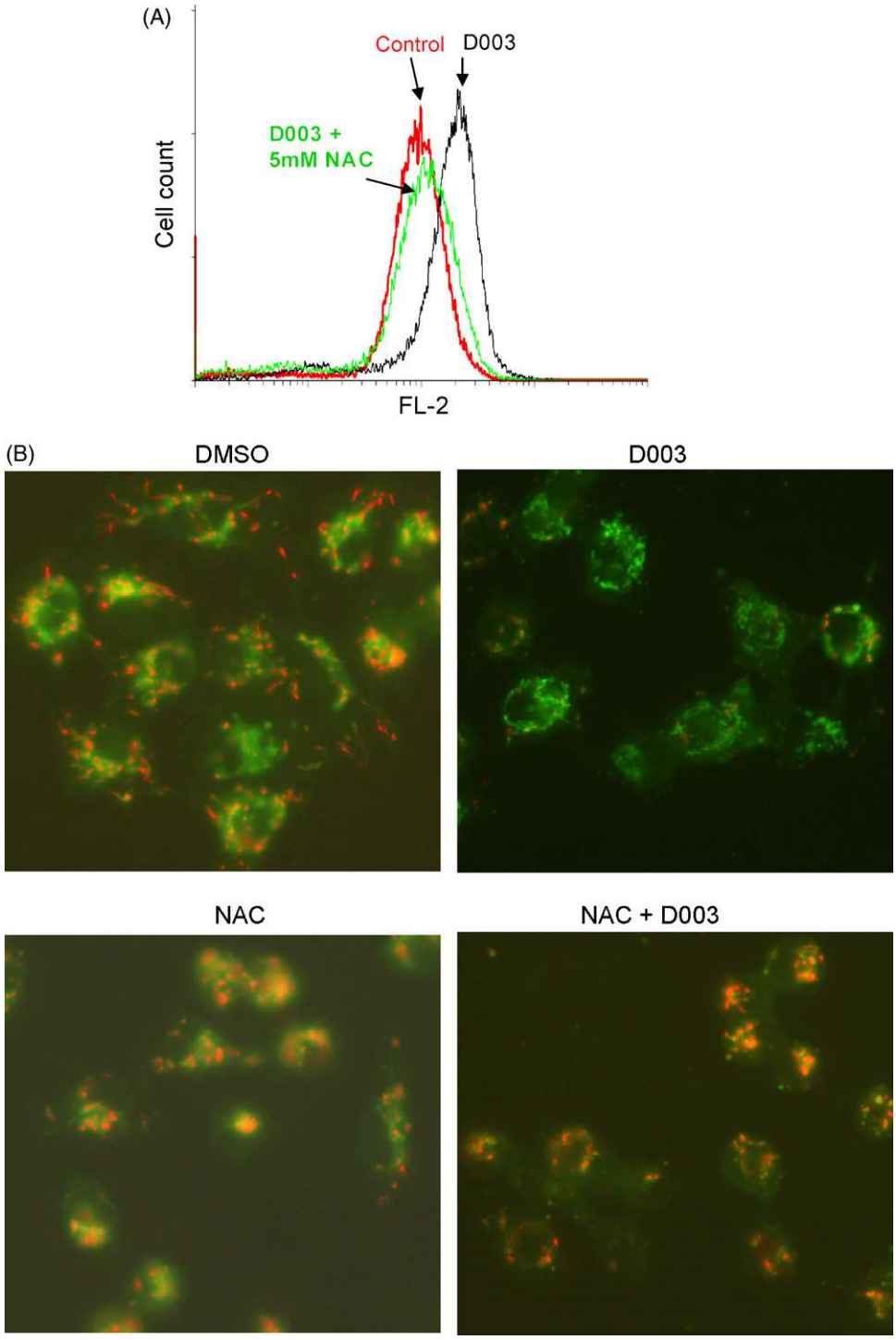

Fig. 5. (A) Change in ROS levels following treatment of the malignant cell line with the avocado chloroform extract (D003). Cells were treated with 30 μg/ml the chloroform extract (D003) for 3 h in the presence or absence of 5 mM NAC. DCF-DA was incubated for 30 min before harvest as a measure of ROS using flow cytometry. (B) The malignant cell line was treated with the chloroform extract (D003) for 16 h in the presence or absence of *N*-acetyl-cysteine (NAC) followed by the mitochondrial potential sensitive dye, JC-1. Red fluorescence indicates normal mitochondria with highly polarized inner member potential, whereas the green fluorescence reflects apoptotic cells with lost mitochondrial potential.

5.1. Avocado chloroform extract-induced apoptosis may be associated with reactive-oxygen species (ROS) production

Redox is a normal physiological process balancing the levels of oxidants and antioxidants. Many cancer cell types produce high levels of ROS, including peroxides, superoxide and nitric oxide, which may contribute to their high proliferation rates, genomic instability and promote invasion by killing adjacent normal cells [68–70]. Tumor cells maintain a delicate balance between oxidants and antioxidant and perturbing this balance may offer an opportunity for therapeutic intervention [70–73]. A number of studies have demonstrated that diverse therapeu-

tic agents induce apoptosis via ROS [74–77]. It is thought that ROS are signals in the initiation of apoptosis via the intrinsic and extrinsic pathways. For example, alpha-lipoic acid, an antioxidant, induces apoptosis via the intrinsic pathway in hepatoma cells by increasing the levels of ROS followed by p53 and Bax and the down regulation of cell cycle regulatory proteins [78]. ROS appears to be required in the activation of oxygenase activity of cytochrome *c*, which oxidizes cardiolipin resulting in the dissociation and release of cytochrome *c* from mitochondria membrane into the cytosol [79]. ROS is an important inducer of apoptosis by activating the FAS/FADD/caspase 8/10 extrinsic pathway [80–84]. Thus, ROS plays an important role in apoptosis via a number of pathways.

In most cases, dietary phytochemicals act as antioxidants to prevent the initial events in cancer. Avocado also contains numerous antioxidant phytochemicals, e.g., persin (**15**, Fig. 1), persenones A (**14**, Fig. 1) and B (**17**, Fig. 1) as described in section 3. Inhibition of nitric oxide generation is mediated by inhibition of inducible NO synthetase (iNOS) [85], which is an inducible enzyme involved in inflammatory tissues and demonstrated to over-express in laboratory cancer cell lines and in vivo tumors. Luteolin (**32**) is reported to inhibit xanthine oxidase-generated superoxide formation and reduce LPS-induced hydroxyl radical formation [86]. In our studies using extracts from the avocado meat, the chloroform extract (D003) selectively increases the levels of ROS in the human oral malignant cell line (Fig. 5A). To further demonstrate the role of ROS in this extract (D003) induced apoptosis, the malignant cell line was pretreated with *N*-acetyl-cysteine (NAC), a scavenger of ROS. NAC greatly reduced the levels of ROS induced by the chloroform extract (D003) coinciding with the inhibition of apoptosis as indicated by normal morphology, lack of PARP cleavage and caspase activation. NAC was also observed to block the loss of mitochondrial membrane potential induced by this extract (D003) (Fig. 5B). Taken together these data suggest that phytochemicals isolated from the avocado: (i) produce ROS that leads to apoptosis mediated through the activation of the FAS/FADD/caspase 8; and (ii) ROS may be a central regulatory molecule activated by the phytochemicals in avocado.

6. Summary and future perspectives

In vitro and in vivo studies are indicating that avocados should be added to the list of fruits as part of a cancer prevention diet. Avocados are a rich source of nutrients as well as cancer preventing phytochemicals. While some of the individual phytochemicals found in avocado have been well characterized, many new uncharacterized phytochemicals are being discovered with potential cancer preventing activity. Studies described here and elsewhere are indicating that combination of phytochemicals as would be found in the whole fruit and extracts from the fruit may even be more effective. It is expected that future studies with avocados will: (i) discover novel cancer preventing phytochemicals; (ii) define molecular mechanisms and targets for growth inhibition and apoptosis; and (iii) lay the foundation for the development of novel diet based preventive strategies in human cancer.

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