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Aliphatic acetogenin constituents of avocado fruits inhibit human oral cancer cell proliferation by targeting the EGFR/RAS/RAF/MEK/ERK1/2 pathway

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Abstract

Avocado (*Persea americana*) fruits are consumed as part of the human diet and extracts have shown growth inhibitory effects in various types of human cancer cells, although the effectiveness of individual components and their underlying mechanism are poorly understood. Using activity-guided fractionation of the flesh of avocado fruits, a chloroform-soluble extract (D003), was identified that exhibited high efficacy towards premalignant and malignant human oral cancer cell lines. From this extract, two aliphatic acetogenins of previously known structure were isolated, compounds **1** [(2*S*,4*S*)-2,4-dihydroxyheptadec-16-enyl acetate] and **2** [(2*S*,4*S*)-2,4-dihydroxyheptadec-16-ynyl acetate]. In this study, we show for the first time that the growth inhibitory efficacy of this chloroform extract is due to blocking the phosphorylation of EGFR (Tyr1173), c-RAF (Ser338), and ERK1/2 (Thr202/Tyr204) in the EGFR/RAS/RAF/MEK/ERK1/2 cancer pathway. Compound **1** and **2** both inhibited phosphorylation of c-RAF (Ser338) and ERK1/2 (Thr202/Tyr204). Compound **2**, but not compound **1**, prevented EGF-induced activation of EGFR (Tyr1173). When compounds **1** and **2** were combined they synergistically inhibited c-RAF (Ser338) and ERK1/2 (Thr202/Tyr204) phosphorylation, and human oral cancer cell proliferation. The present data suggest that the potential anticancer activity of avocado fruits is due to a combination of specific aliphatic acetogenins that target two key components of the EGFR/RAS/RAF/MEK/ERK1/2 cancer pathway.

Keywords

Avocado fruit; $CHCl_3$-soluble extract; aliphatic acetogenins (alkanols); EGFR; ERK1/2; oral cancer

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Conflict of interest
None.

1. Introduction

Extracellular-signal-regulated kinases (ERKs) are highly conserved protein kinases that are involved in a variety of cellular activities including proliferation, differentiation, survival, and death. The epidermal growth factor receptor (EGFR), a cell membrane tyrosine kinase, can activate multiple downstream cell signaling pathways including the RAS/RAF/MEK/ERK1/2 pathway [1, 2]. Over-activation of EGFR/RAS/RAF/MEK/ERK1/2 pathway is considered an etiological factor in human cancer, which contributes to cancer development, metastasis, and resistance to chemotherapy [1, 2]. Therefore, targeting EGFR, RAS, RAF, MEK, and ERK1/2 has become a promising approach in cancer therapy and prevention. Small molecule inhibitors targeting specific kinases in this pathway have been developed and tested in clinical trials. However, outcomes using single kinase inhibitors have not been promising [3]. One possible explanation is that the EGFR/RAS/RAF/MEK/ERK1/2 pathway can be alternatively activated by amplification or mutation in any signaling molecule in the pathway, such as of EGFR, RAS, RAF, and other growth factor receptors [4], which makes it difficult to use target specific small molecule inhibitors for cancer prevention.

Fruits and vegetables contain multiple anticancer phytochemicals, which have been extensively explored as a cancer prevention approach. Studies have shown that avocado fruit extracts exhibit antiproliferative effects in human cancer cell lines [5]. Our published studies have reported that a chloroform-soluble extract of the mesocarp of avocado fruit, designated as D003, induces apoptosis in human oral cancer cells via regulation of ROS at high concentrations (≥30 μg/ml) and inhibits cell proliferation at lower concentrations (5–20 μg/ml) by a non-apoptotic mechanism [5, 6]. Avocado fruit contains several phytochemicals that potentially may have anticancer activity. It appears that certain carotenoids in an avocado acetone extract increase protein expression of tumor suppressor gene *p27* and induce cell cycle arrest at G_2/M phases in prostate cancer cell lines [7]. Aliphatic acetogenins, also known as "alkanols", are a class of compounds almost exclusively isolated from avocado. Among these compounds, persenone A was found to inhibit nitric oxide synthase and cyclooxygenase in a mouse macrophage cell line [8] and reduced nitric oxide and superoxide generation in inflammatory leukocytes [9]. Compounds **1** [(2*S*,4*S*)-2,4-dihydroxyheptadec-16-enyl acetate] and **2** [(2*S*,4*S*)-2,4-dihydroxyheptadec-16-ynyl acetate] showed inhibition of acetyl CoA carboxylase [10]. To date, no biological evaluation has been carried out in terms of the potential anticancer or chemopreventive activities of compounds **1** and **2**. In the present study, we have investigated the potential role of the EGFR/RAS/RAF/MEK/ERK1/2 pathway in the antiproliferative effect of the avocado and have identified the $CHCl_3$-soluble extract D003 and compounds **1** and **2** as targeting this pathway.

2. Materials and methods

2.1. General experimental procedures

Optical rotations were measured on a Perkin-Elmer 241 automatic polarimeter. NMR spectroscopic data were recorded at room temperature on a Bruker Avance DRX-400 spectrometer with tetramethylsilane (TMS) as an internal standard. HRESIMS and ESIMS were obtained on a 3-Tesla Finnigan FTMS-2000 Fourier transform mass spectrometer. Column chromatography was performed with Sephadex LH-20 (Supelco, Bellefonte, PA), 65–250 or 230–400 mesh Si gel (Sorbent Technologies, Atlanta, GA). Analytical thin-layer chromatography was conducted on precoated 250 μm thickness Partisil Si gel 60 F_{254} glass plates (Whatman, Clifton, NJ), and preparative thin-layer chromatography was performed on precoated 20 cm × 20 cm, 500 μm or 1000 μm thickness Partisil Si gel 60 F_{254} glass plates (Whatman, Clifton, NJ).

2.2. Extraction and isolation

Hass avocado fruit (*Persea americana* L.; Lauraceae) was kindly provided by California Avocado Commission. Avocado chloroform extract (code name: D003, 1.1 g) was prepared from avocado mesocarp (871 g) as previously described [5, 6]. This chloroform extract was chromatographed over a silica gel column using a gradient solvent system of CH_2Cl_2-MeOH (100:0 to 0:100), to yield eight sub-fractions (D003F01-F08). These sub-fractions were tested in an *in vitro* cell growth inhibition assay using normal and cancerous human oral epithelial cell lines. The most active fraction toward the premalignant and malignant cell lines, D003F02, was subjected to a silica gel column chromatographic purification using a solvent mixture of hexanes-ethyl acetate for elution, to afford ten additional sub-fractions (D003F0201-F0210). From combined subfractions D003F0203 and D003F0204 which had similar growth inhibition and selectivity activities toward cancerous cell lines, compounds **1** (8.0 mg) and **2** (7.5 mg), were purified according to our published protocols, in yields of 0.0092% and 0.0086%, respectively [11–13]. The identities of these two compounds were established as (2*S*,4*S*)-2,4-dihydroxyheptadec-16-enyl acetate (**1**) and (2*S*,4*S*)-2,4-dihydroxyheptadec-16-ynyl acetate (**2**) respectively, by comparing their spectroscopic data with those published in the literature [14].

2.3. Cell lines

The human oral cancer cell line 83-01-82CA, MEK overexpressing cell line 83-01-82CA/MEK_{CA} and its control cell line 83-01-82CA/GFP have been described previously [15].

2.4. Growth inhibition assay

Methylene blue staining was used to estimate cell proliferation, as previously described [16]. Human oral cancer cell line 83-01-82CA was seeded (3×10^3) into 96-well plates in 100 μl of growth medium. After cells had attached overnight, they were incubated with extract D003, compound **1**, or compound **2** for 72 h.

2.5. Western Blot Analysis

Following treatment of cells, proteins were harvested using SDS buffer and processed according to our previously published protocol [15]. Primary antibody specific for the following proteins were used: phospho-EGF receptor (Tyr1173, 53A5, 1:500), EGF receptor (D38B1, 1:500), phospho-c-RAF (Ser338) (56A6, 1:1000), phospho-p44/42 MAPK (Thr202/Tyr204) (E10, 1:1,000), from Cell Signaling Technology, and α-tubulin (Ab-1, 1:1000) from Oncogene Research Products (Darmstadt, Germany).

3. Results

3.1. The chloroform extract (D003) of avocado fruit inhibits EGFR and its downstream c-RAF/ERK1/2

The human oral cancer cell line 83-01-82CA was incubated with D003 extract for 48 and 72 h in full growth medium. The results showed that the D003 extract down-regulated total protein levels of EGFR, phosphorylated c-RAF (Ser338) and ERK1/2 (Thr202/Tyr204) in a concentration-dependent manner (Fig. 1), while EGFR phosphorylation (Tyr1173) was undetectable in D003 extract-treated and -untreated samples using this protocol. These data suggest that avocado D003 extract targets EGFR and its downstream RAF and ERK1/2 pathway.

3.2. Enhanced ERK1/2 rescues D003 extract-induced growth inhibition

ERK1/2 is one of the most important downstream effectors of EGFR through the EGFR/RAS/RAF/MEK/ERK1/2 pathway. To investigate the importance of ERK1/2 in D003

extract-induced growth inhibition, ERK1/2 was overactivated by expressing a constructively active MEK, an upstream activator of ERK1/2, in the human oral cancer cell line, 83-01-82CA (83-01-82CA/MEK_{CA}) [15]. As shown in Fig. 2, overactivation of ERK1/2 markedly protected human oral cancer cells from D003 extract-induced growth inhibition, with an IC_{50} value of 18.8 μg/ml in the 83-01-82CA/MEK_{CA} cell line versus 8.6 μg/ml in the control 83-01-82CA/GFP cell line. These data suggest that inhibition of EGFR/RAS/RAF/MEK/ERK1/2 pathway is important in the D003 avocado extract-induced growth inhibition in human oral cancer cells.

3.3. Avocado aliphatic acetogenin constituents inhibit EGFR and its downstream ERK1/2

From the D003 extract we isolated and identified two previously known acetogenin constituents, compounds **1** and **2** (Fig. 3A). Growth inhibition assays indicated that both these components exhibited a concentration-dependent growth inhibition of the 83-01-82CA cell line (GI_{50} value of 38 μM for compound **1** and 60 μM for compound **2**) (data not shown). Next, we determined whether these two components target EGFR/RAS/RAF/MEK/ERK1/2 pathway. Fig. 3B shows that both compounds **1** and **2** markedly inhibited the phosphorylation levels of c-RAF (Ser338)/ERK1/2 (Thr202/Tyr204) in the absence of EGFR ligand EGF. In the presence of EGF, compound **1** (30–120 μM) did not block EGF-induced phosphorylation of EGFR (Tyr 1173), but inhibited the activation of c-RAF and ERK1/2 in its downstream pathway. In contrast, compound **2** (30–120 μM) completely blocked EGF-induced EGFR phosphorylation (Tyr 1173) and inhibited phosphorylation of downstream c-RAF (Ser338)/ERK1/2 (Thr202/Tyr204). These data indicate that compounds **1** and **2** target the EGFR/RAS/RAF/MEK/ERK1/2 pathway by different mechanisms. Since human oral cancer cell line 83-01-82CA harbors a mutant and constructively active H-RAS [17], these data also suggest that compound **1** may target RAS/RAF and compound **2** may target both EGFR and RAS/RAF.

3.4. Compounds 1 and 2 synergistically inhibit human oral cancer cell proliferation

Since compounds **1** and **2** target the EGFR/RAS/RAF/MEK/ERK1/2 pathway through different mechanisms, we determined their combined effect on the growth and EGFR signaling in human oral cancer cells. As shown in Fig. 4A, a synergistic inhibitory effect (CI < 1.0) on growth inhibition was observed when compounds **1** and **2** were combined in the ratios of 1:1, 1:2 and 2:1 from 2.5–20 μM. Next, the combination effect on the levels of EGFR and down-downstream RAF/ERK1/2 was investigated. After 24 h in serum-free media, 83-01-82CA cells were treated with EGF together with compounds **1** and **2** individually at 5, 10 and 20 μM or in combination at 1:1 ratios. As shown in Fig. 4B, 5 μM **1** or **2** alone exhibited no inhibitory effect on EGF-induced phosphorylation of EGFR (Tyr1173) and c-RAF (Ser338) and a mild suppression of phosphorylation of ERK1/2. Their combination had no effect on the phosphorylation of EGFR (Tyr1173), but significantly inhibited the phosphorylation of c-RAF (Ser338) and ERK1/2 (Thr202/Tyr204). When concentrations were increased to ≥10 μM, compound **2**, but not **1**, inhibited phosphorylation of EGFR (Tyr1173). Even though these two compounds alone partly inhibited EGF-induced phosphorylation of c-RAF (Ser338) and ERK1/2 (Thr202/Tyr204), their combination significantly ($p<0.05$) blocked the phosphorylation of c-RAF (Ser338) and ERK1/2 (Thr202/Tyr204). Together, these data suggest that compounds **1** and **2** target different molecules in the EGFR/RAS/RAF/MEK/ERK1/2 pathway and their combination leads to a synergistic inhibitory effect on cell proliferation that may be mediated by a synergistic inhibition of the EGFR/RAS/RAF/MEK/ERK1/2 pathway.

4. Discussion

Fruits and vegetables have shown promise in cancer prevention. Numerous phytochemicals isolated from a variety of plants have been identified as selective cancer growth inhibitors and/or apoptotic agents. Avocado fruit meat only recently has been identified as containing cancer-preventing phytochemicals not found in other fruits and vegetables [5]. In the present study, we have identified and characterized the underlying mechanisms of a bioactive chloroform-soluble extract (D003) isolated from avocado mesocarp and two pure components (**1** and **2**) that selectively target human oral cancer cells. It has been shown that the extract D003 selectively inhibited the growth of human oral cancer cell lines by targeting the EGFR/RAS/RAF/MEK/ERK1/2 pathway. We have demonstrated further that two aliphatic acetogenins (**1** and **2**), isolated and purified from this extract, act synergistically to inhibit the EGFR/RAS/RAF/MEK/ERK1/2 pathway via different mechanisms.

Previous studies have shown that the potential anticancer activity of avocado fruit mesocarp extracts may be related to the inhibition of cell growth via the induction of tumor suppressor p21, and reduction of the cell-cycle proteins, cyclins D1, B1, A [5]. Our previously published data indicated that that avocado extract D003 induces apoptosis via modulation of mitochondrial ROS production at concentrations ≥30 μg/ml [5]. In the present study, it has been shown that the avocado extract D003 at non-apoptotic concentrations (≤20 μg/ml) and two isolated constituents [(2*S*,4*S*)-2,4-dihydroxyheptadec-16-enyl acetate (**1**) and (2*S*, 4*S*)-2,4-dihydroxyheptadec-16-ynyl acetate (**2**)] inhibit EGFR and its downstream RAF/MEK/ERK1/2 pathway in human oral cancer cells. To our knowledge, this is the first report of EGFR as a target of the avocado D003 extracts and these two isolated compounds. Inhibition of EGFR by avocado extract D003 appears to be due to reducing both the total amount of EGFR protein and its activation. Among the multiple function-regulating residues in the EGFR molecule, the following sites are involved in the activation of the downstream RAS: phosphorylation at Tyr1148 and Tyr1173 provides a docking site for SHC scaffold protein [18]; while phosphorylation at Tyr 1068 results in the binding of Grb2 and SOS proteins leading to the activation of RAS by converting inactive GDP-bond RAS to active GTP-bound RAS [19, 20]. We tested the modulation of Tyr1173 and Tyr 1068, which show similar changes after stimulation with EGF and treatment with avocado D003 extract and the two isolated components (**1** and **2**). Therefore, it is proposed that the avocado D003 extract inhibits the activation of EGFR at least via these two tyrosine residues related to RAS activation. Additional studies are required to investigate how D003 extract and the isolated components inhibit these tyrosine residues. We suspect that the modulation of the phosphorylation status of EGFR is involved in the immediate inhibition while reduction of total protein may result from a lengthy exposure.

Compounds **1** and **2**, are aliphatic acetogenin derivatives, reported so far only from *P. americana*. The only structural difference between these two compounds is that compound **1** possesses a terminal alkene, while compound **2**, has a terminal alkyne. However, this structural difference caused a marked change in their interaction with cellular EGFR pathway. Our data indicates that compound **2** inhibits the EGFR/RAS/RAF/MEK/ERK1/2 pathway at two different levels: i) inhibiting EGFR activation (Tyr1173) by EGF; and ii) suppressing activation of c-RAF (Ser 338). Since the 83-01-82CA cell line contains a constructively active H-RAS mutation at codon 12 (Gly to Val) and compound **2** inhibits both EGFR and c-RAF, we reason that inhibition of c-RAF by compound **2** is via inhibition of RAS or RAF, independent of upstream signal EGFR. Unlike compound **2**, compound **1** does not inhibit EGFR phosphorylation at Tyr 1173. It does however inhibit c-RAF activity (Ser 338), either directly on c-RAF or upstream on RAS.

The different mechanisms of compounds **1** and **2** in targeting the EGFR/RAS/RAF/MEK/ERK1/2 pathway provide a rationale for compound combination in cancer prevention and treatment. Our data indicate that on combining these compounds at low concentrations they act synergistically to inhibit the proliferation of human oral cancer cells. Considering that the EGFR/RAS/RAF/MEK/ERK1/2 pathway may be activated in cancer cells by either EGFR or RAS [4, 21–23], targeting multiple molecules in the pathway simultaneously may provide more effective prevention and treatment than using specific inhibitors targeting only one of the molecules in the EGFR pathway. In fact, recent studies suggest that RAS mutation may be responsible for tumor resistance to EGFR inhibitors in patients [4, 24, 25]. Therefore, providing a double hit on a critical cancer pathway such as EGFR/RAS/RAF/MEK/ERK1/2 by phytochemicals like those found in avocado fruit could lead to more effective method of cancer prevention.

Acknowledgments

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References

1. Okamoto I. Epidermal growth factor receptor in relation to tumor development: EGFR-targeted anticancer therapy. FEBS J. 2010; 277:309–315. [PubMed: 19922468]
2. Mitsudomi T, Yatabe Y. Epidermal growth factor receptor in relation to tumor development: EGFR gene and cancer. FEBS J. 2010; 277:301–308. [PubMed: 19922469]
3. Dancey J, Sausville EA. Issues and progress with protein kinase inhibitors for cancer treatment. Nat Rev Drug Discov. 2003; 2:296–313. [PubMed: 12669029]
4. Wheeler DL, Dunn EF, Harari PM. Understanding resistance to EGFR inhibitors-impact on future treatment strategies. Nat Rev Clin Oncol. 2010; 7:493–507. [PubMed: 20551942]
5. Ding H, Chin YW, Kinghorn AD, D'Ambrosio SM. Chemopreventive characteristics of avocado fruit. Semin Cancer Biol. 2007; 17:386–394. [PubMed: 17582784]
6. Ding H, Han C, Guo D, Chin YW, Ding Y, Kinghorn AD, D'Ambrosio SM. Selective induction of apoptosis of human oral cancer cell lines by avocado extracts via a ROS-mediated mechanism. Nutr Cancer. 2009; 61:348–365. [PubMed: 19373608]
7. Lu QY, Arteaga JR, Zhang Q, Huerta S, Go VL, Heber D. Inhibition of prostate cancer cell growth by an avocado extract: role of lipid-soluble bioactive substances. J.Nutr.Biochem. 2005; 16:23–30. [PubMed: 15629237]
8. Kim OK, Murakami A, Takahashi D, Nakamura Y, Torikai K, Kim HW, Ohigashi H. An avocado constituent, persenone A, suppresses expression of inducible forms of nitric oxide synthase and cyclooxygenase in macrophages, and hydrogen peroxide generation in mouse skin. Biosci.Biotechnol.Biochem. 2000; 64:2504–2507. [PubMed: 11193428]
9. Kim OK, Murakami A, Nakamura Y, Kim HW, Ohigash H. Inhibition by (−)-persenone A-related compounds of nitric oxide and superoxide generation from inflammatory leukocytes. Biosci.Biotechnol.Biochem. 2000; 64:2500–2503. [PubMed: 11193427]
10. Hashimura H, Ueda C, Kawabata J, Kasai T. Acetyl-CoA carboxylase inhibitors from avocado (Persea americana Mill) fruits. Biosci.Biotechnol.Biochem. 2001; 65:1656–1658. [PubMed: 11515553]
11. Wall ME, Wani MC, Brown DM, Fullas F, Oswald JB, Josephson FF, Thornton NM, Pezzuto JM, Beecher CWW, Farnsworth NR, Cordell GA, Kinghorn AD. Effect of tannins on screening of plant extracts for enzyme inhibitory activity and techniques for their removal. Phytomedicine. 1996; 3:281–285.
12. Kinghorn AD, Farnsworth NR, Soejarto DD, Cordell GA, Swanson SM, Pezzuto JM, Wani M, Wall ME, Oberlies NH, Kroll D, Kramer RA, Rose W, Vite G, Fairchild C, Peterson R, Wild R. Novel strategies for the discovery of plant-derived anticancer agents. Pharm.Biol. 2003; 41:53–67.
13. Kinghorn AD, Carcache-Blanco EJ, Chai H-B, Orjala J, Farnsworth NR, Soejarto DD, Oberlies NH, Wani MC, Kroll DJ, Pearce CJ, Swanson SM, Kramer RA, Rose WC, Fairchild CR, Vite GD,

Emanuel S, Jarjoura D, Cope FO. Discovery of anticancer agents of diverse natural origin. Pure Appl Chem. 2009; 81:1051–1053. [PubMed: 20046887]

14. Domergue F, Helms GL, Prusky D, Browse J. Antifungal compounds from idioblast cells isolated from avocado fruits. Phytochemistry. 2000; 54:183–189. [PubMed: 10872209]
15. Ding H, Han C, Guo D, Wang D, Chen CS, D'Ambrosio SM. OSU03012 activates Erk1/2 and cdk2/cyclin A leading to the accumulation of cells in the S-phase and apoptosis. Int.J.Cancer. 2008; 123:2923–2930. [PubMed: 18798257]
16. Han C, Ding H, Casto BC, Stoner GD, D'Ambrosio SM. Inhibition of the growth of premalignant and malignant human oral cell lines by extracts and components of black raspberries. Nutr Cancer. 2005; 51:207–217. [PubMed: 15860443]
17. Lee H, Li D, Prior T, Casto BC, Weghorst CM, Shuler CF, Milo GE. Ineffectiveness of the presence of H-ras/p53 combination of mutations in squamous cell carcinoma cells to induce a conversion of a nontumorigenic to a tumorigenic phenotype. Cell Biol Toxicol. 1997; 13:419–434. [PubMed: 9352120]
18. Zwick E, Hackel PO, Prenzel N, Ullrich A. The EGF receptor as central transducer of heterologous signalling systems. Trends Pharmacol.Sci. 1999; 20:408–412. [PubMed: 10577253]
19. Quilliam LA, Huff SY, Rabun KM, Wei W, Park W, Broek D, Der CJ. Membrane-targeting potentiates guanine nucleotide exchange factor CDC25 and SOS1 activation of Ras transforming activity. Proc.Natl.Acad.Sci.U.S.A. 1994; 91:8512–8516. [PubMed: 8078913]
20. Aronheim A, Engelberg D, Li N, al-Alawi N, Schlessinger J, Karin M. Membrane targeting of the nucleotide exchange factor Sos is sufficient for activating the Ras signaling pathway. Cell. 1994; 78:949–961. [PubMed: 7923364]
21. Nagatsuka H, Ishiwari Y, Tsujigiwa H, Nakano K, Nagai N. Quantitation of epidermal growth factor receptor gene amplification by competitive polymerase chain reaction in pre-malignant and malignant oral epithelial lesions. Oral Oncol. 2001; 37:599–604. [PubMed: 11564582]
22. Ishitoya J, Toriyama M, Oguchi N, Kitamura K, Ohshima M, Asano K, Yamamoto T. Gene amplification and overexpression of EGF receptor in squamous cell carcinomas of the head and neck. Br.J.Cancer. 1989; 59:559–562. [PubMed: 2713242]
23. Bos JL. Ras oncogenes in human cancer: A review. Cancer Res. 1989; 49:4682–4689. [PubMed: 2547513]
24. Allegra CJ, Jessup JM, Somerfield MR, Hamilton SR, Hammond EH, Hayes DF, McAllister PK, Morton RF, Schilsky RL. American Society of Clinical Oncology provisional clinical opinion: testing for KRAS gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. J.Clin.Oncol. 2009; 27:2091–2096. [PubMed: 19188670]
25. Linardou H, Dahabreh IJ, Kanaloupiti D, Siannis F, Bafaloukos D, Kosmidis P, Papadimitriou CA, Murray S. Assessment of somatic k-RAS mutations as a mechanism associated with resistance to EGFR-targeted agents: a systematic review and meta-analysis of studies in advanced non-small-cell lung cancer and metastatic colorectal cancer. Lancet Oncol. 2008; 9:962–972. [PubMed: 18804418]

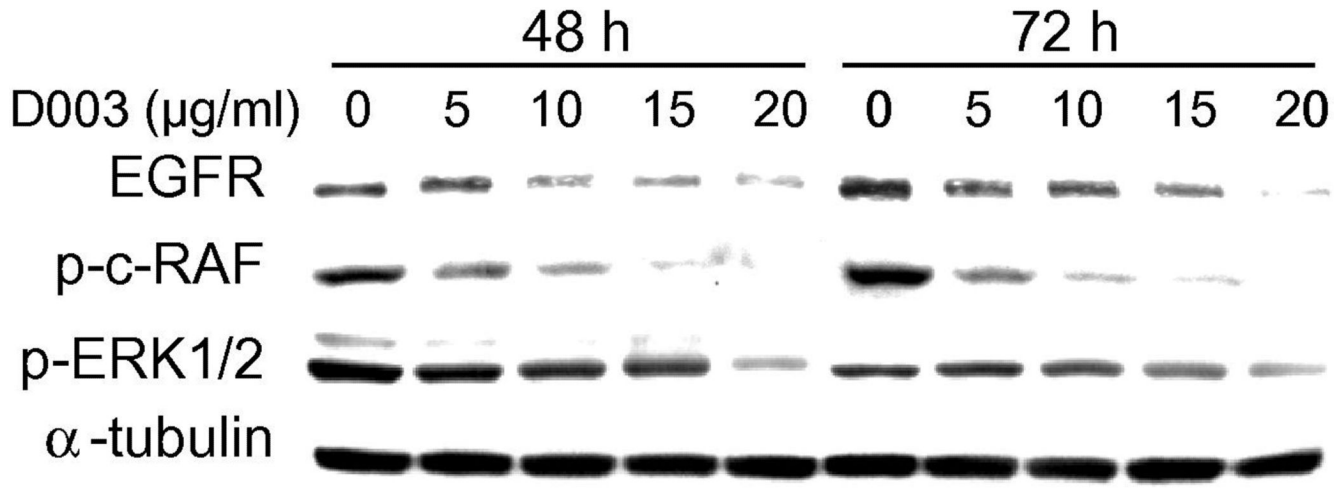


Fig. 1.

The avocado D003 extract inhibits the activity of EGFR and its downstream c-RAF/ERK1/2. The human oral cancer cell line 83-01-82CA was seeded in 100 mm cell culture dishes in growth medium. After overnight incubation, cells were treated with 5–20 μg/ml D003 extract for 48 and 72 h. Equal amounts of protein were analyzed by Western blot using specific antibodies against EGFR and phosphorylated c-RAF (Ser338) (p-c-RAF) and ERK1/2 (Thr202/Tyr204) (p-ERK1/2). α-Tubulin was used as a loading control.

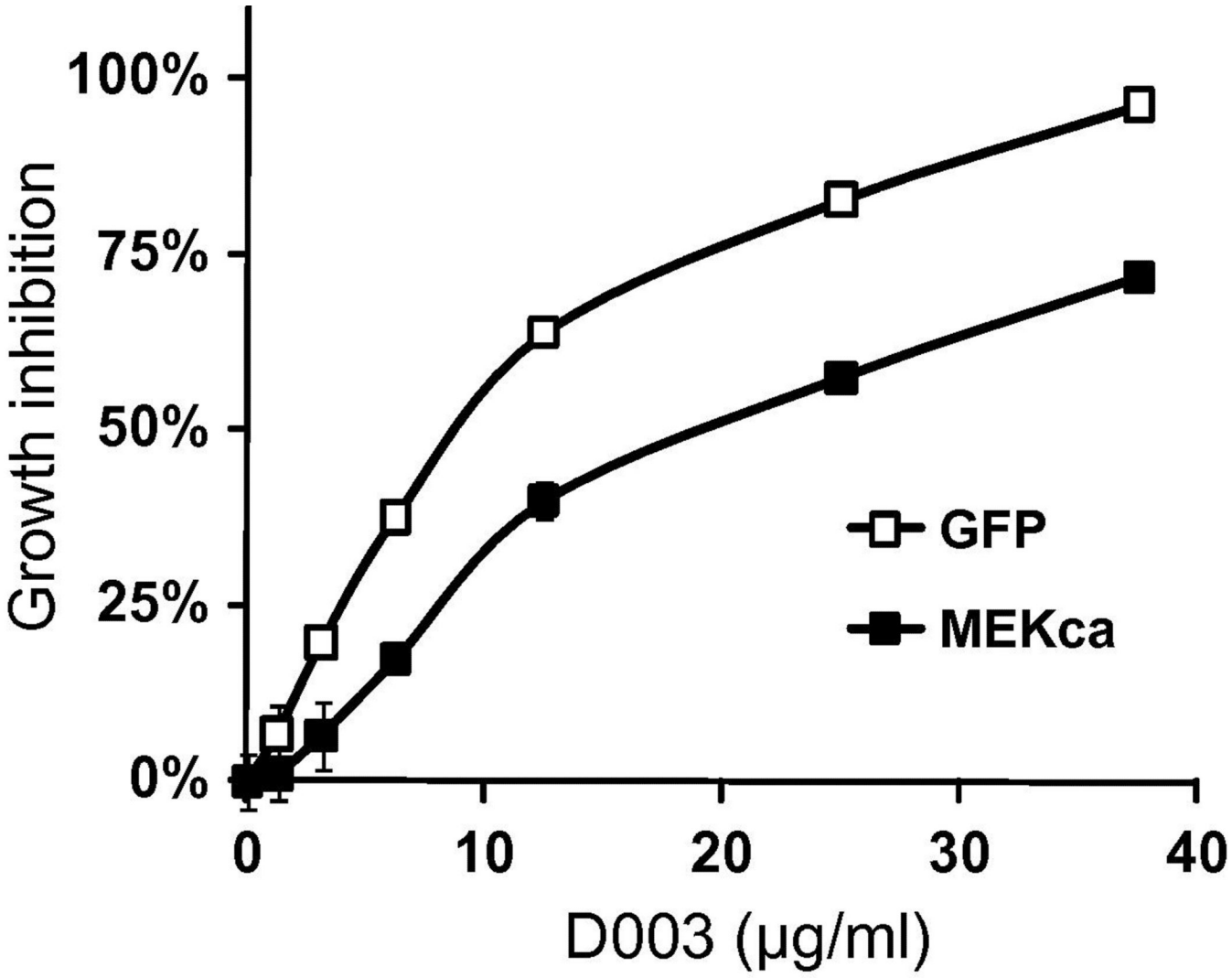
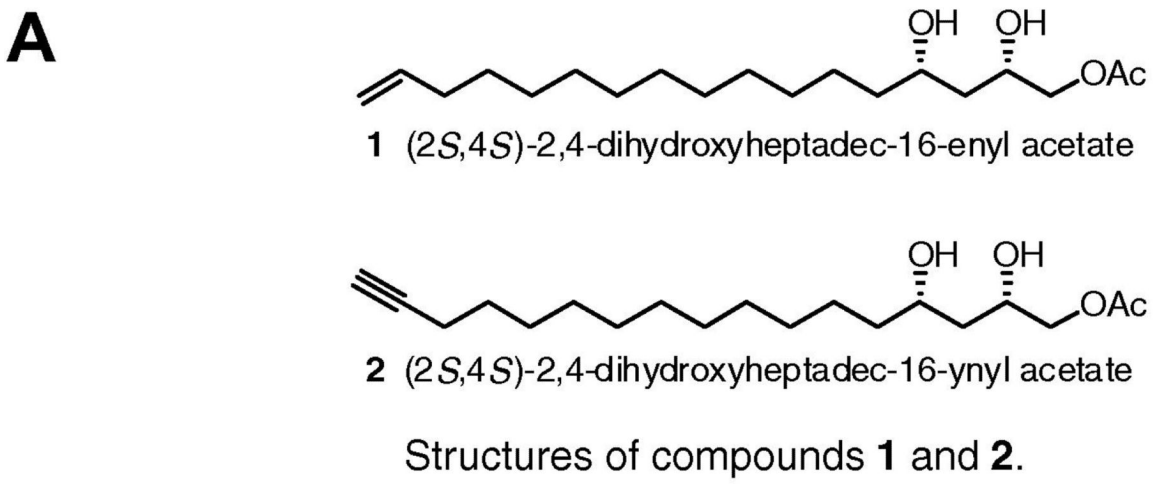


Fig. 2.

Over-activation of MEK/ERK1/2 partially reverses avocado D003 extract-induced growth inhibition. The 83-01-82CA cells expressing constructively active MEK (MEK_{CA}) and the empty vector (GFP) are described in the Materials and Methods. Cells were treated 0–37.5 μg/ml avocado D003 extract in growth medium for 72 h. Relative cell numbers were determined by the methylene blue assay. The data were normalized to the control treated with vehicle DMSO.



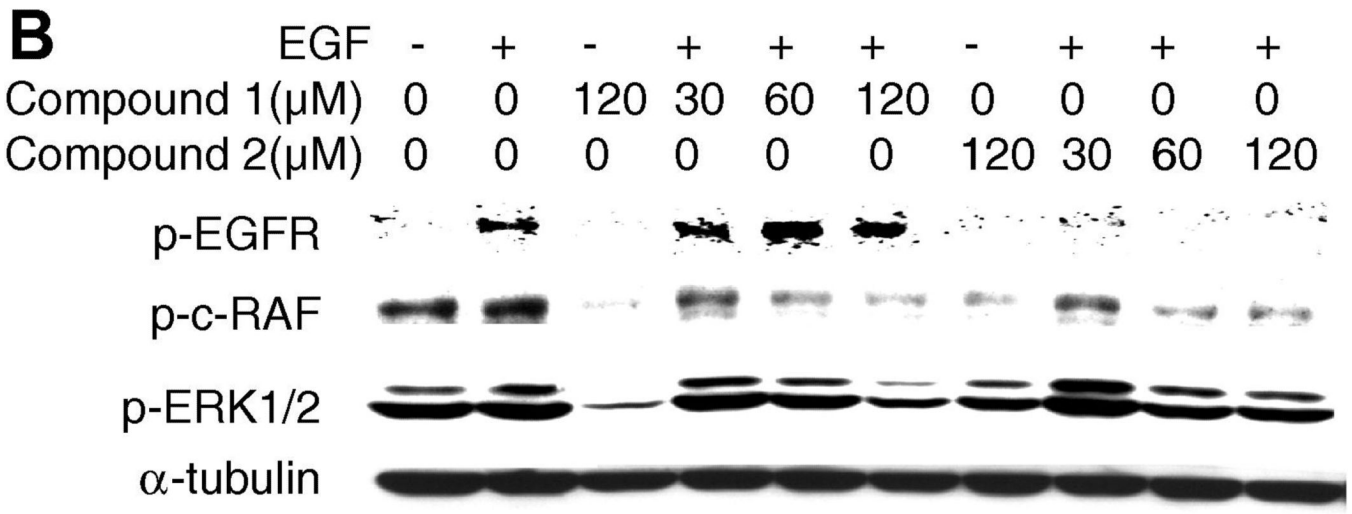


Fig. 3.

Compounds **1** and **2** isolated from avocado D003 extract inhibit the activity of EGFR and its downstream c-RAF and ERK1/2. ***A***, Structure of compounds **1** and **2**. ***B***, The 83-01-82CA cells were seeded in 100 mm cell culture dishes in growth medium. After overnight incubation, the medium was replaced with serum-free medium and cells were starved for 24 h. Cells were then incubated with 30–120 μM compounds **1** and **2** for 1 h followed by 10 ng/ml EGF for 10 min. Proteins were harvested and protein levels of phosphorylated forms of EGFR (Tyr 1173) (p-EGFR), c-RAF (Ser 338) (p-c-RAF) and ERK1/2 (Thr202/Tyr204) (p-ERK1/2) were determined by Western blotting. α-Tubulin was used as a loading control.

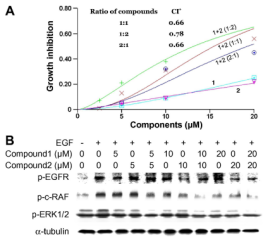


Fig. 4.

The combination of compounds **1** and **2** act synergistically to inhibit cancer cell growth. ***A***, Growth inhibition of the 83-01-82CA cell line by individual (0, 5, 10 and 20 μM) and combined concentrations (ratio of 1:1, 1:2, and 2:1) of compounds **1** and **2**. Cells were incubated for 72 h and relative cell numbers were determined by methylene blue staining. Values are normalized to vehicle DMSO controls. A combination index (CI) was determined using Calcusyn software (inset). * Combination results are synergistic when CI is <1. ***B***, The 83-01-82CA cells were seeded and starved as in Fig. 3B, followed by incubation with compounds **1** and **2** for 1 h using the same concentration scheme (1:1) as in Fig. 4*A* and then incubated with 10 ng/ml EGF for 10 min. Proteins were harvested and protein levels of phosphorylated forms of EGFR (Tyr 1173) (p-EGFR), c-RAF (Ser 338) (p-c-RAF) and ERK1/2 (Thr202/Tyr204) (p-ERK1/2) were determined by Western blotting. α-Tubulin was used as a loading control.