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Oleuropein, a non-toxic olive iridoid, is an anti-tumor agent and cytoskeleton disruptor

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Abstract

Oleuropein, a non toxic secoiridoid derived from the olive tree, is a powerful antioxidant and anti angiogenic agent. Here, we show it to be a potent anti cancer compound, directly disrupting actin filaments in cells and in a cell free assay. Oleuropein inhibited the proliferation and migration of advanced grade human tumor cell lines in a dose responsive manner. In a novel tube disruption assay, Oleuropein irreversibly rounded cancer cells, preventing their replication, motility, and invasiveness; these effects were revers ible in normal cells. When administered orally to mice that developed spontaneous tumors, Oleuropein completely regressed tumors in 9 12 days. When tumors were resected prior to complete regression, they lacked cohesiveness and had a crumbly consistency. No viable cells could be recovered from these tumors. These observations elevate Oleuropein from a non toxic antioxidant into a potent anti tumor agent with direct effects against tumor cells. Our data may also explain the cancer protective effects of the olive rich Med iterranean diet.

Keywords: Mediterranean diet; Actin; Matrigel; Polyphenol; Elenolic acid; Hydroxytyrosol; Chemotherapy; Oncology; Cancer; Chemoprevention

Large epidemiological studies encompassing several countries showed that the Mediterranean region had a significantly reduced all-cause death rate [1 5]. This reduction was mainly due to decreased incidence of cardiovascular disease and cancer. The protective effect was attributed to the Mediterranean diet, rich in fruits and vegetables, whole grains, and olive products [5]. The Lyon Heart Study, a large randomized clinical trial, demonstrated that a modified diet based on that of the Mediterranean reduced total deaths by 56% and decreased the cancer risk by 61% at a 4-year follow up [1]. Other diets, similarly rich in fruits and vegetables but lacking the olive component, were not equally protective [3,4,6,7]. Evidence for the protective role of olives was also derived by comparing olive oil consumption in the Mediterranean countries to that of the United States. Mediterranean populations consume 20 times more olive oil than Americans; correspondingly, their cancer risk is at least half [3]. More direct evidence for the protective role of olive oil against cancer has been recently published by Filik and Ozyilkan [8].

Olive oil is rich in oleic acid and other monounsaturated fats with various biological actions. In recent studies, diets containing 15% olive oil significantly reduced chemically induced pre-cancerous lesions in rat breast and colon [9 11]. Even though the authors anticipated that a similar proportion of soy oil would be equally protective, this did not occur, suggesting that the chemo-preventive capacity of olive oil was not due to unsaturated fatty acids. A similar study determined that a high corn oil diet allowed the development of malignant adenocarcinomas in rats, whereas a high olive oil diet did not [12]. In addition to fatty acids, olives and olive oil are rich in powerful antioxidants such as polyphenols and flavonoids with diverse biological activities [13,14].

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Numerous lines of evidence demonstrate that antioxidants protect against DNA damage, a major step in oncogenic processes [15]. Oleuropein is the most abundant of the phenolic compounds in olives [16]. It actively scavenges reactive oxygen [17] and nitrogen species [18] as well inducing the production of nitric oxide in macrophages [19]. In addition, we have previously shown that Oleuropein is anti-angiogenic [20]. Even though the above qualities advance it as a major candidate for explaining the protective role of the Mediterranean diet, in this paper we present further evidence that Oleuropein has direct anti-tumor effects.

Materials and methods

Cell culture. Normal human skin fibroblasts (NL Fib) and the following human advanced grade tumor cell lines were purchased from the American Type Culture Collection, ATCC (Manassas, VA) and cultured according to the supplier's instructions: LN 18, poorly dif ferentiated glioblastoma; TF 1a, erythroleukemia; 786 O, renal cell adenocarcinoma; T 47D, infiltrating ductal carcinoma of the breast pleural effusion; MCF 7, mammary gland adenocarcinoma, pleural effusion; RPMI 7951, malignant melanoma of the skin lymph node metastasis; and LoVo, colorectal adenocarcinoma supraclavicular region metastasis. All cells were used within 10 passages after arrival. Cell culture reagents were purchased from Invitrogen Life Technolo gies (Carlsbad, CA). Cell culture flasks and sterile pipettes were ob tained from USA Scientific (Ocala, FL). Nalgene sterile filtration units were from Fisher Scientific (Hampton, NH). All cultures were main tained in a 37 °C, 5% CO_2 atmosphere.

Cell proliferation. Approximately 5×10^3 cells (TF 1a; 786 O, T 47D, RPMI 7951, LoVo, and NL Fib) were seeded in triplicate wells of 96 well plates in 100 μl of their respective growth medium with increasing doses of purified Oleuropein (Indofine Chemical, Hillsbor ough, NJ). Cell proliferation was determined on day 5 using the CellTiter MTS cell proliferation kit (Promega, Madison, WI) accord ing to the manufacturer's instructions. Absorbance at 490 nm was measured with a Bio Tek Instruments microplate reader. Background readings (medium ± Oleuropein without cells + CellTiter reagents) were subtracted from the experimental readings.

β Glucosidase treatment. Five hundred microliters of a 1% solution of Oleuropein in fibroblast tissue culture medium was incubated with ±25U of purified β glucosidase from almonds (Sigma Chemical, St. Louis, MO) for 2 days at 37 °C. Control and enzymatically treated Oleuropein was then diluted to a final concentration of 0.025% for the cell proliferation assay as described above.

Cell migration. Fibroblasts and adherent tumor cell lines (LN 18, RPMI 7951, 786 O, and T 47D) were grown to confluence in 24 well tissue culture plates. The monolayer was wounded vertically using the wooden end of a sterile cotton swab stick. For more extensive damage, monolayers were also wounded in the shape of a cross with a sterile 1 ml pipette tip. Detached cells were removed by washing with phos phate buffered saline (PBS) and the remaining monolayer was covered with 500 μl of growth medium with or without 0.01% Oleuropein. Original edges were marked for reference. Alternatively, some experi ments measuring radial migration were carried out as follows: approximately 1×10^5 cells in a 5 μl volume were deposited as a drop in the center of a 24 well plate and incubated for 4 6 h at 37 °C to allow for adherence. The wells were subsequently washed with PBS to re move free floating cells and covered with 500 μl of growth medium with or without 0.01% Oleuropein. In all cases, cell migration was monitored daily by phase contrast microscopy (Swift Instruments International). At the conclusion of the experiment, cells were washed with PBS, fixed with 100% methanol at room temperature for 15 min, stained with Mayer's hematoxylin (Sigma) for 5 min, and rinsed with distilled water prior to photography with a Kodak MDS100 digital camera.

Reversibility of cell rounding. Fibroblasts and adherent tumor cells were seeded at approximately 5×10^4/well in a 24 well plate. Oleu ropein was added to their respective growth medium to a final con centration of 0.01% followed by 24 h incubation. The cells were carefully washed at least five times with 2 ml of growth medium per wash and the medium was replaced with normal growth medium. Cells were photographed 48 h later.

Cell invasion. Falcon cell culture inserts (3 μm pore size) were cooled to −20 °C on top of their corresponding 24 well plates (Becton Dickinson Discovery Labware, Bedford, MA) and overlaid with a 3 mm layer of ice cold Matrigel (Becton Dickinson) according to the manufacturer's instructions. Matrigel solidification occurred at 37 °C for at least 1 h. Subsequently, 100 μl of 0.5% fetal calf serum (FCS) containing culture medium with approximately 2×10^5 LoVo colo rectal cancer cells was added to the insert, whereas normal culture medium containing 10% FCS was added to the bottom well as a chemo attractant. After a 4 to 6 h incubation, 11 μl of a 1% Oleu ropein solution was added to the top chamber of duplicate wells. Cells were observed daily by phase contrast microscopy and photographed. On day 10, the filters were removed from the cell culture inserts, fixed in methanol, and stained with Mayer's hematoxylin.

Tube disruption assay. Triplicate wells of a previously frozen 96 well tissue culture plate were coated with 50 μl of Matrigel. After solidification at 37 °C, approximately 1×10^5 RPMI 7951 melanoma cells were added in 100 μl of 0.5% FCS containing culture medium. After a 24 h incubation to allow for the formation of vascular like tubes, 11 μl of a 1% Oleuropein solution was added. Some Oleuropein treated wells also received D glucose (Sigma) to a final concentration of 3.5% (weight/volume). Cells were observed hourly by phase contrast microscopy and photographed as described above.

Immunofluorescence. Approximately 1×10^3 breast cancer cells (MCF 7) were seeded in a TiterTek II culture chamber slide (USA Scientific) and allowed to adhere for 24 h at 37 °C. The culture medium was changed to 0.5% FCS containing medium with or without 0.01% Oleuropein. Some Oleuropein treated wells had been pre incubated in 0.5% FCS medium containing 3.5% D glucose for 5 min. After a 2 h incubation, the slide was washed with PBS, fixed in ice cold methanol for 15 min, and allowed to dry. After rehydration in PBS, the slide was blocked with 5% normal rabbit serum in PBS and processed for immunofluorescence as previously described [21]. Secondary antibody was a rabbit anti mouse conjugated to TRITC (Sigma), 1:1000 in PBS, whereas the primary antibody was a mouse monoclonal against pan actin (LabVision; Freemont, CA) at 2 μg/ml in PBS. To determine non specific staining, we used the irrelevant FITC rabbit anti goat IgG at 1:1000 (Sigma). Final images were derived from merging the FITC and TRITC filtered images using Adobe Photoshop software.

Purified actin filament disruption. This assay was a modification of the actin polymerization kit protocol (Cytoskeleton; Denver, CO). All solutions were prepared according to the manufacturer's instructions. G actin was incubated for 1 h at RT with "G" buffer ± 0.01% solution of Oleuropein in "G" buffer. For visualization, 5 μl of G actin solu tion ± Oleuropein was deposited on a clean, poly lysine coated glass slide together with 1 μl TRITC phalloidin (Sigma) dissolved in meth anol according to the manufacturer's instructions. After the addition of 5 μl of actin polymerization buffer, slides were incubated for 1 min at RT, cover slipped, and photographed under a fluorescence microscope.

Animal studies. Three to four mice of the same gender were housed in individual cages with food and water ad libitum. They were in a temperature and humidity controlled environment with filtered air and regular light/dark cycles. A proprietary, house established inbred strain of Swiss albino mice that spontaneously develops soft tissue sarcomas was used (H_2RC, Orange, CA). This mouse strain was

developed by inbreeding six generations arising from the F1 progeny of a male founder mouse that developed a spontaneous tumor without human intervention. Approximately 25% of male and female mice developed tumors around 1 year of age. If necessary to improve tumor visualization, hair was removed from the tumor area using a depilatory cream. After the tumors reached a visible diameter of at least 2 cm, mice were divided into two groups: five were assigned to the control (untreated) group, whereas 15 were assigned to the treatment group. Fourteen mice received 1% Oleuropein in their drinking water, which they consumed ad libitum. Pilot experiments had already shown that this was the maximum concentration of Oleuropein that could be used, since higher concentrations were too bitter and the mice refused to drink. Tumor size was monitored daily. On day 5, one of the Oleu ropein treated mice had its tumors removed for examination and development of primary tumor cell lines. The remaining animals in the treatment group continued to drink the Oleuropein containing water until their tumors completely regressed, usually in 9 12 days. Untreated mice exhibited 100% mortality by the 10th day, whereas all 12 Oleuropein treated mice remain(ed) tumor free for the remainder of their lifespan. One mouse was given a 500 µl intraperitoneal injection of a 10% Oleuropein solution in Hanks' phosphate buffered saline every 12 h for 2 days. On the third day, the tumor was resected, examined, and photographed.

Statistical analysis. Paired Student's *t* test was conducted using GraphPad Prism 4 software.

Results

Cell proliferation

The effect of Oleuropein on cell proliferation was assessed on normal human fibroblasts and tumor cell lines derived from advanced-grade human tumors (TF-1a; 786-O, T-47D, RPMI-7951, and LoVo). Cells were incubated with increasing doses of Oleuropein (0.005 0.025%). After 5 days, cell numbers were measured using a tetrazolium salt-based assay (Fig. 1). Optical densities are shown rather than normalized values to demonstrate the differential growth rates of the cell lines with respect to one another. All cell lines were growth-inhibited in a dose-dependent manner by the addition of Oleuropein. However, in the case of 786-O (renal cell adenocarcinoma) and normal fibroblasts, only the highest concentration was effective.

β-Glucosidase treatment of Oleuropein

In order to investigate whether the glucose moiety in Oleuropein affected its biological activity, we removed it with β-glucosidase treatment for 2 days prior to its use in the cell proliferation assay as above. When normal fibroblasts were counted on day 5, β-glucosidase-treated Oleuropein was less effective in inhibiting cell proliferation than the non-treated control (Fig. 2).

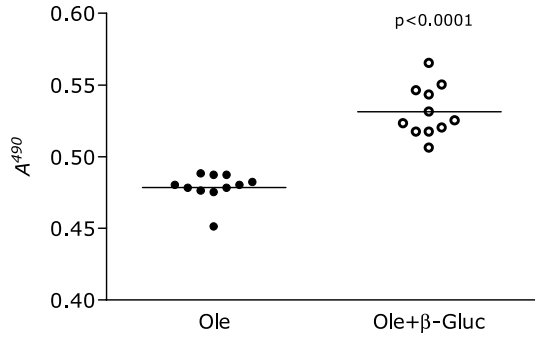

Fig. 2. β Glucosidase decreases anti proliferative activity of Oleurop ein. Oleuropein was incubated with ±β glucosidase for 48 h at 37 °C and added to approx. 5×10^3 normal fibroblasts. Cell proliferation was measured on day 5 with the CellTiter kit. Bars indicate mean of absorbance readings from four experiments.

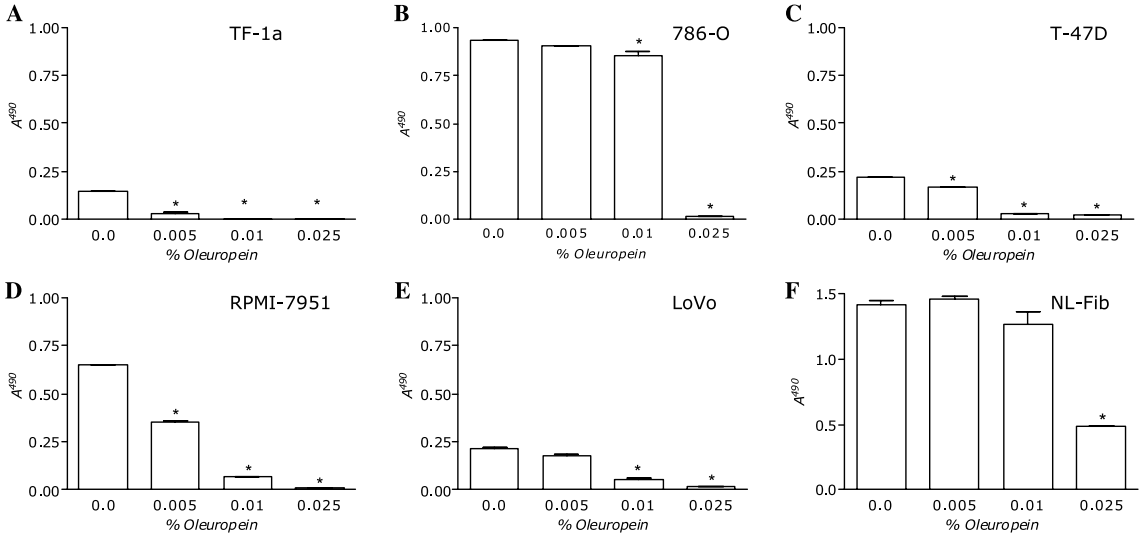

Fig. 1. Oleuropein inhibits cell proliferation. Approximately 5×10^3 human tumor cells (A E) or normal fibroblasts (F) were seeded in 96 well plates with increasing doses of Oleuropein. Cell proliferation was measured on day 5 with the CellTiter kit. Bars indicate means ± standard deviation of absorbance readings from two experiments in triplicate. $*p < 0.05$ vs. control.

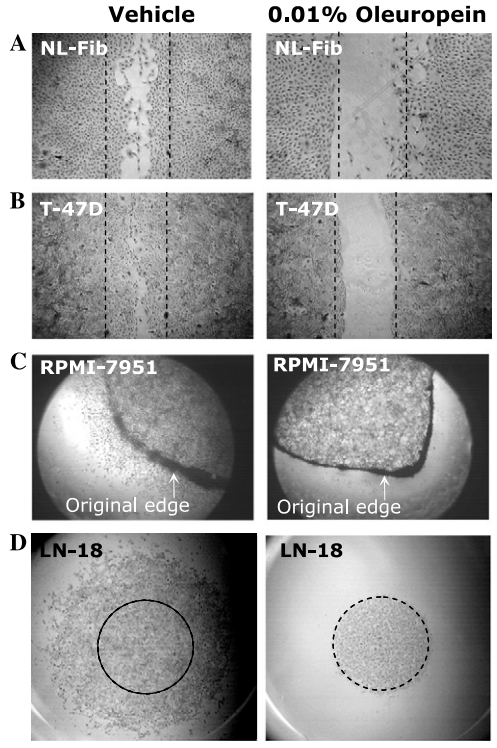

Fig. 3. Oleuropein inhibits cell migration. Confluent monolayers were wounded vertically (standard wound assay, A and B) or using a cross pattern (C). Alternatively, cells were seeded in one spot (radial migration assay, D). In all cases, detached cells were washed away and Oleuropein was added to 0.01% in fresh culture medium. Cells were fixed and stained on day 3. Dotted lines mark original edges. Magnification: 100× (A,B); 40× (C); and 20× (D).

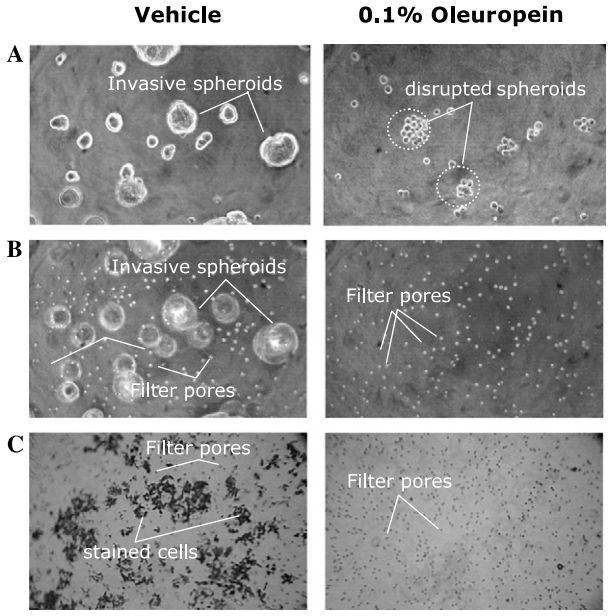

Fig. 4. Oleuropein inhibits tumor cell invasion. LoVo colorectal carcinoma cells in 0.5% serum medium ± Oleuropein were plated inside transwell inserts coated with undiluted Matrigel. The bottom culture chamber contained 10% serum medium as a chemo attractant. (A) The migrating tumor cell spheroids on day 5, with the camera focusing on the cells. (B) The same field as (A) with the camera focusing on the filter. On day 10, filters were removed, fixed, and stained. (C) The stained filters at the conclusion of the experiment. Magnification: 100×.

Cell migration

Using three variations of cell migration assays, we show that 0.01% Oleuropein completely inhibits cell motility in all cell lines. Representative photographs are shown in Fig. 3. Panels A and B show the inhibition of normal fibroblasts and T-47D breast cancer cell migration by Oleuropein using the traditional monolayer wound assay. To better define the wound edge from the migrating cells, we modified this assay and increased the severity of the wound by making a cross pattern; this allowed the edge of the monolayer to curl, making it more visible. Using RPMI-7951 melanoma cells, we clearly show their migratory inhibition by Oleuropein (Fig. 3C). In panel D, we demonstrate a similar effect of Oleuropein on the radial migration of LN-18 glioblastoma cells.

Cell invasion

Using the highly invasive, spheroid-forming colon carcinoma cell line LoVo, we found that 0.1% Oleuropein completely blocked the invasion of tumor cells through a thick, undiluted Matrigel layer to the other side of the filter membrane (Fig. 4C). During this experiment, we noticed that Oleuropein-treated spheroids were disrupted and were incapable of moving through the matrix (Figs. 4A and B). By focusing on the filter, it is clear that the tumor spheroids in the Oleuropein-treated wells are far from the membrane whereas in the control, they appear close by. Interestingly, Oleuropein-treated spheroids contained rounded cells and appeared less cohesive (Fig. 4A).

Tube disruption

To study the kinetics of cell rounding, we modified the existing tube-formation assay on Matrigel. Cancer cell invasion assays in Boyden chambers use soft Matrigel (1 2 mg of protein/ml); however, tube-formation assays use hard Matrigel (9 12 mg protein/ml) to provide node anchoring. The tube-formation assay is used to study pro/anti angiogenic effects of substances on vascular endothelial cells. This is possible because vascular endothelial cells seeded on hard Matrigel undergo a programmed series of morphological changes, including tube formation and retraction. Endothelial cell tube formation on hard Matrigel occurs by cell motility and cell cell contact. The tubes, however, are not stable and retract into clumps within 24 48 h [22]. Taking advantage of the "vascular mimicry" behavior of melanoma cells [23], we show that the human melanoma cell

line RPMI-7951 can also form tubes on hard Matrigel (Fig. 5). Unlike the vascular endothelial cell tubes, melanoma tubes collapse by retraction in 1 week. In certain cases, they never retract and cells invade the matrix (not shown). When added to the tubing phase of the assay, 0.1% Oleuropein disrupted the tubes in situ by rounding the cells and preventing tube retraction (Fig. 5). This process is relatively fast, occurring within 2 h; this contrasts with tube retraction, which occurs within 1 week. Since Oleuropein has a glucose moiety, we explored the possibility that it could enter the cell through the glucose transport system. Interestingly, co-incubation with excess D-glucose decreased the ability of Oleuropein to induce cell rounding, suggesting this as a possible mechanism of entry.

Reversibility of cell rounding

We next investigated whether the Oleuropein-induced cell rounding was reversible by extensively washing the Oleuropein-treated melanoma tubes and subsequent culturing in normal growth medium. Even after thorough washing and incubation in normal medium for 10 days, tumor cells remained immobile, round, and did not proliferate (not shown). Because of the possibility that Matrigel could act as a sponge to sequester Oleuropein, we decided to repeat the experiment on Matrigel-free cultures of RPMI-7951 melanoma cells as well as normal cells. We found that 0.01% Oleuropein rounded both normal and tumor cells; however, only the normal cell rounding was reversible after washing. Within 48 h, normal cells flattened out and again became mobile (Fig. 6). On the contrary, cancer cells remained immobilized after washing even after 1 week of culture in normal growth medium (not shown).

Actin filament disruption

Given that Oleuropein-induced cell rounding occurred within 2 h, we hypothesized that Oleuropein could be affecting the cytoskeleton. Adherent breast carcinoma cells (MCF-7) grown on chamber slides were treated with 0.01% Oleuropein for 2 h, fixed, and stained with a pan-actin monoclonal antibody. As shown in Fig. 7A, Oleuropein treatment dramatically disrupts the organization of actin filaments within the cells. This disruption coincided with the 2-h time frame for cell rounding observed previously. Co-incubation with excess D-glucose decreased the ability of Oleuropein to disrupt the cytoskeleton, suggesting the involvement of the glucose transport system. Fig. 7B demonstrates that Oleuropein directly disrupted actin filaments in a cell-free assay. The nature and kinetics of the disruption is currently under investigation.

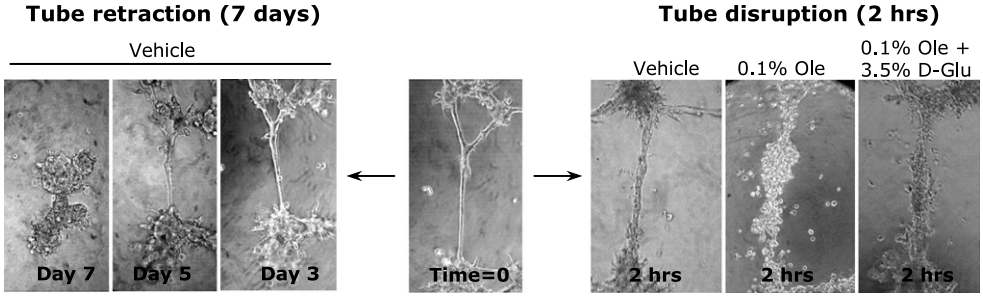

Fig. 5. Oleuropein disrupts melanoma tubes on Matrigel. RPMI 7951 human melanoma cells were seeded on undiluted Matrigel and allowed to form tubes. Left panels show the normal tube retraction over time. Right panels demonstrate the effect of Oleuropein (Ole) on tube structure. Addition of 0.1% Oleuropein induced cell rounding within 2 h. Excess D glucose partially inhibited Oleuropein's disruptive action. Magnification: 100×.

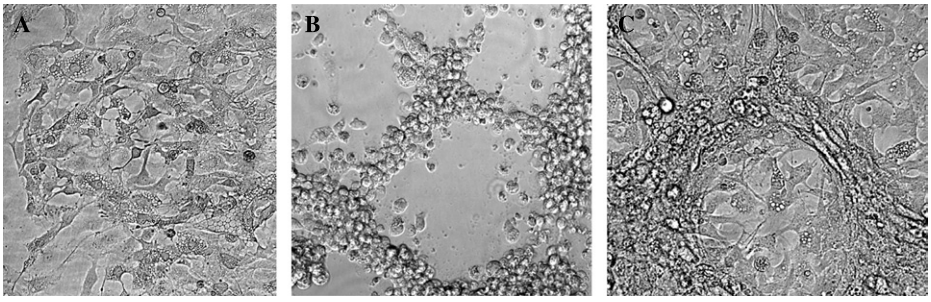

Fig. 6. Oleuropein induced cell rounding is reversible in normal cells. Confluent monolayers of normal skin cells (A) were treated with 0.01% Oleuropein. After 2 h, the cells were photographed (B) and thoroughly washed. The same field was re photographed after 48 h of incubation in normal growth medium (C). Magnification: 200×.

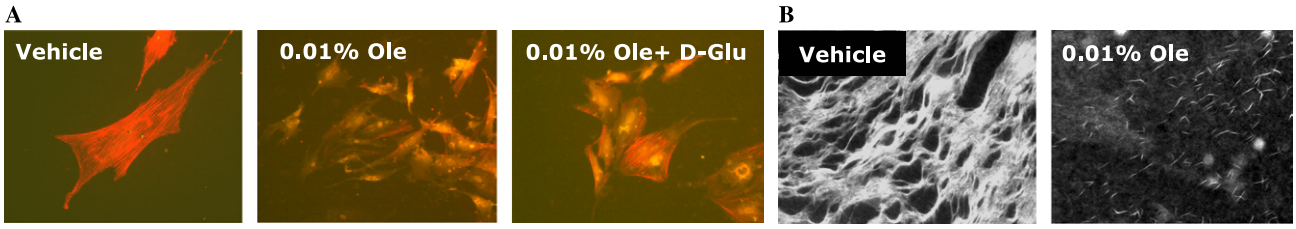

Fig. 7. Oleuropein disrupts actin filaments. (A) Immunofluorescence. Adherent MCF 7 cells were incubated for 2 h ± 0.01% Oleuropein (Ole) ± excess D glucose (D Glu). Actin filaments were stained with a pan actin antibody and TRITC anti mouse IgG. (B) Purified actin. G actin (pre incubated for 1 h ± 0.01% Oleuropein) was polymerized on a glass slide. Filaments were visualized with TRITC phalloidin. Magnification: 200×.

Animal studies

Having established Oleuropein's efficacy in vitro, we proceeded to determine its anti-tumor action in vivo. Using Swiss albino mice that spontaneously develop soft tissue sarcomas, we determined that 1% Oleuropein in the drinking water (consumed ad libitum) induced dramatic tumor regression (Table 1, Fig. 8). Pilot experiments had already shown that this was the maximum concentration of Oleuropein that could be used, since higher concentrations were too bitter and the mice refused to drink. Oleuropein induced complete tumor regression in 10/11 mice and partial regression in one animal. It seems that Oleuropein had a similar effect on mice bearing single (mouse B) or multiple (mouse A) tumors but the increased tumor load may have slightly delayed the time to complete regression (9 vs. 12 days). The complete regression of such large tumors (>2 cm in diameter) within 12 days was surprising and reproducible (Table 1). Interestingly, after the tumors regressed, the region where the tumor had been located was sunken, with excess skin folds and a dark underlying area (Fig. 8). Within a week, the area became normal (not shown). After tumors regressed, Oleuropein treatment was discontinued and mice were returned to regular drinking water. All mice remained tumor-free for the remainder of their lifespan (Table 1). It is unknown whether this tumor-free state was due to the Oleuropein treatment or to the nature of the animal model itself. It is possible that tumor development was age-dependent. Because this mouse strain arose through a natural occurrence without human intervention, the exact mechanism by which the tumors develop is unclear. Because all untreated animals die within 2 weeks of tumor appearance, it was not possible to determine whether tumors would have continued to appear if the animal had survived.

In order to obtain samples for analysis, we resected incompletely regressed tumors on day 5 of Oleuropein treatment (not shown). We noticed that the Oleuropein-treated mice had tumors of a non-cohesive, crumbly consistency, unlike tumors from non-treated animals, which were more fibrous and solid.

Table 1
Experimental outcomes and survival for control and Oleuropein treated mice

Mouse number	Ole treatment (9 12 days)	Experimental outcome	Survival (months)
1	None	Increased tumor	0
2	None	Increased tumor	0
3	None	Increased tumor	0
4	None	Increased tumor	0
5	None	Increased tumor	0
6	Oral (ad libitum)	Complete regression	18+
7	Oral (ad libitum)	Complete regression	14
8	Oral (ad libitum)	Complete regression	18+
9	Oral (ad libitum)	Complete regression	18+
10	Oral (ad libitum)	Complete regression	12
11	Oral (ad libitum)	Complete regression	18
12	Oral (ad libitum)	Complete regression	12
13	Oral (ad libitum)	Complete regression	15+
14	Oral (ad libitum)	Complete regression	14+
15	Oral (ad libitum)	Complete regression	14+
16	Oral (ad libitum)	Partial regression	3
17	Oral (ad libitum)	Early resection	N/A
18	IP injection	Early resection	N/A

Ole, Oleuropein. IP, intraperitoneal injection. "+" denotes the mouse was still alive at the time of manuscript submission.

Fig. 8. Oleuropein induces tumor regression in vivo. Mice bearing spontaneous tumors were administered 1% Oleuropein in their drinking water. Some mice had multiple tumors (represented by mouse A), whereas others bore a single tumor mass (represented by mouse B). After 9 12 days of treatment, tumors had completely regressed.

We attempted to develop primary tumor cell lines from these Oleuropein-treated tumors without success. Therefore, it was necessary to shorten the treatment to 2 days. Since we were unsure whether the mice would drink enough in a 2-day period, we injected Oleuropein intraperitoneally. After 2 days, the tumor was removed and analyzed (Fig. 9). This tumor appeared heterogeneous (Fig. 9A, inset) and was still vascularized. Parts of the tumor, especially those near blood vessels, exhibited a crumbly consistency (Fig. 9B) containing clusters of rounded cells (Fig. 9C). The small fragments that separated from the main tumor mass during dissection were mostly composed of rounded cell clusters (Fig. 9D) and resembled the disrupted tumor spheroids we observed in the Matrigel invasion and tube-disruption assays (Figs. 4 and 5). Intriguingly, we also observed a crystalline accumulation in the crumbly parts of the tumor (Fig. 9D, arrowhead). These crystals were similar in shape and size to Oleuropein crystals in a saturated solution (not shown) but their identity remains undetermined.

Discussion

Since the 1800s, the bitter component in olives was used in humans against malaria-induced fevers. Later studies showed that it also possessed anti-microbial, anti-viral, and anti-fungal activities [24,25]. In 1960, Panizzi and Oriente [26] succeeded in isolating Oleuropein from the olive bitter fraction. Its chemical structure was definitively elucidated in 1970 by Inouye et al. [27] using Oleuropein purified from the Japanese privet tree *Ligustrum japonicum*. Purified Oleuropein and its metabolite elenolic acid also possessed anti-microbial, anti-viral, and anti-fungal properties (reviewed in [28]). Further experimentation showed that it was a powerful antioxidant [29,30] as well as a hypotensive [31,32] and hypoglycemic agent [32,33]. It also exerted protective effects against heart disease and had immunoregulatory actions [19,34,35].

Some studies were undertaken to determine the toxicity of Oleuropein and its two main metabolites (hydroxytyrosol and elenolic acid); all were found to be completely non-toxic in several animal species

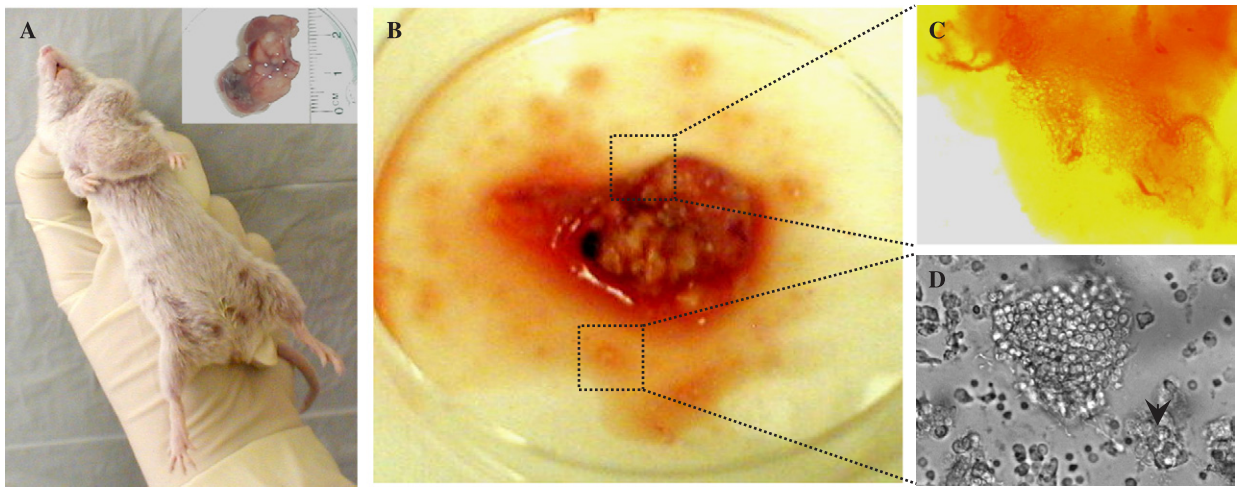

Fig. 9. Oleuropein induces tumor cell rounding in vivo. One mouse (A) was given a 500 ml intraperitoneal injection of a 10% Oleuropein solution in Hanks' phosphate buffered saline every 12 h for 2 days. The tumor (A D) was resected and measured on day 3 (A, inset). The tumor was heterogeneous and had crumbly regions (B) containing clusters of rounded cells (C,D) and crystals (arrowhead). Magnification: (B), 10×; (C) and (D), 100×.

[31,36,37]. In acute toxicity studies for Oleuropein, no lethality or adverse effects were observed in mice even when it was administered at doses as high as 1000 mg/kg, thus an LD_{50} could not be determined [31]. Also, we have previously shown that injection of Oleuropein into fertilized chicken eggs did not interfere with normal embryonic development [20]. Because developing embryos are extremely sensitive to toxicity, these results provide even stronger support for the safety of Oleuropein. Importantly, the breakdown products of Oleuropein, hydroxytyrosol and elenolic acid, exhibited no toxicity even at doses as high as 2000 mg/kg of body weight [36,37]. Even though several human studies have been conducted with olive extract or its polyphenolics showing no adverse effects (reviewed in [29,34,38 40]), more systematic efforts are needed to examine the safety of Oleuropein in humans.

Given that Oleuropein is a non-toxic compound with numerous beneficial properties, we wondered if it had effects on cancer cells. In this paper, we demonstrate its direct anti-tumor activities. We show that Oleuropein inhibits cell growth, motility, and invasiveness. We also show that it induces cell rounding, a phenomenon that has been linked to the disruption of the actin cytoskeleton [41]. We indeed have shown that Oleuropein disrupts the actin cytoskeleton of living cells within 2 h as well as directly disrupting purified actin filaments in a cell-free assay (Fig. 7). In cells, this effect was partially counteracted by the concomitant addition of glucose to the cultures. Given the fact that removal of Oleuropein's glucose moiety with β-glucosidase decreases its anti-proliferative activity (Fig. 2), it is possible that glucose transporters (GLUTs) may facilitate the diffusion of Oleuropein into the cell. There are currently 12 GLUT proteins with various glucose affinities and tissue-specific distributions [42]. It has been reported that human malignancies are characterized by elevated glucose uptake and utilization based on enhanced expression of multiple GLUT isoforms [43]. For example, GLUT1 and/or GLUT3 mRNA and/or protein was increased in cancers of the cervix, thyroid, prostate, breast, and colon [43 47]. Thus, it is likely that cancer cells that overexpress specific GLUT proteins would be more likely to uptake Oleuropein. This might explain the differential sensitivity to Oleuropein of the various cancer cell lines we tested (leukemia > melanoma > colon and breast > kidney) shown in Fig. 1. The fact that most normal cells have low or no expression of certain GLUTs [46] may explain the reversibility of cell rounding in normal but not in cancer cells, after Oleuropein treatment (Fig. 6). In spite of the above, it is likely that Oleuropein also enters the cell by other routes. This is supported by the fact that competition with excess glucose as well as β-glucosidase treatment did not completely abolish the biological activity of Oleuropein (Figs. 2, 5, and 7).

Interestingly, the Oleuropein-induced cell rounding observed in our in vitro experiments was also detected in vivo. In our animal studies, tumor regression occurred quite rapidly, with large (>2 cm diameter) tumors completely disappearing within 9 12 days. This is quite unique among chemotherapeutic agents, since most of them do not induce complete regression in such a short time. This rapid effect of Oleuropein on tumors could be explained by its almost immediate effect on the cytoskeleton, which we observed in vitro. To determine whether cell rounding also occurred in vivo, we examined partially regressed tumors. Even a short, 2-day treatment with Oleuropein was sufficient to induce cell rounding within the tumor itself without having obvious effects on the vasculature (Fig. 9). Our data suggest that the principal anti-tumor mechanism of Oleuropein in vascularized tumors involved the direct disruption of tumor cells.

To combat cancer, medicine has relied on toxic compounds [48]. Most therapies do not discriminate between normal and cancer cells, leading to toxicity and unwanted side effects. In recent years, major efforts have focused on identifying and testing anti-angiogenic compounds as cancer therapeutics. Although this anti-cancer approach appeared very promising when first proposed, it has not been as successful as envisioned [49]. It appears that anti-angiogenic therapies would be more successful if used in chemoprevention rather than on established tumors. Recent developments show that powerful survival mechanisms are triggered in cancer cells under hypoxic conditions [49]. This may explain the limited success of most anti-angiogenic therapies, in which the surviving patients experience more aggressive tumor growth after the initial treatment [50]. These arguments are better understood in the context of the tumor microenvironment. In the nascent tumor containing less than 300 cells, there is minimal genetic variability. At this stage, a tumor is more vulnerable to drugs because all the cells are equally susceptible, due to the clonal nature of the tumor. It is at this point that anti-angiogenic therapies (chemo-preventive) would be most effective by preventing further growth of the tumor. Unfortunately, existing technology is not yet able to detect a clump of 300 cancer cells and patients go untreated. As the tumors become vascularized, the number of cells increases exponentially and tumor cell genetic variability becomes extensive. It is at this later stage that current technology can detect tumors and cancer therapies are thus initiated. If an anti-angiogenic therapy is given when the tumor is already highly vascularized, a selection process ensues in the tumor leading to the growth of hypoxia-resistant cells, which can survive and proliferate in this new environment [49,51]. Because Oleuropein quickly targets tumor cells prior to its targeting of tumor vasculature, a selection process driven by hypoxia may not take place.

We propose that Oleuropein represents a new class of anti-cancer compounds, which targets multiple steps in cancer progression. As an antioxidant, it may protect cells from incurring genetic damage leading to oncogenesis. As an anti-angiogenic agent, it can prevent tumor progression. Finally by directly inhibiting cancer cells, it can lead to tumor regression. The unique combination of these properties in a single molecule should elevate Oleuropein from a dietary component into an active cancer drug worthy of human studies.

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