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Multiple antimelanoma potential of dry olive leaf extract

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Various constituents of the olive tree (*Olea europaea*) have been traditionally used in the treatment of infection, inflammation, prevention of chronic diseases, cardiovascular disorders and cancer. The anticancer potential of dry olive leaf extract (DOLE) represents the net effect of multilevel interactions between different biologically active compounds from the extract, cancer cells and conventional therapy. In this context, it was of primary interest to evaluate the influence of DOLE on progression of the highly malignant, immuno- and chemoresistant type of skin cancer—melanoma. DOLE significantly inhibited proliferation and subsequently restricted clonogenicity of the B16 mouse melanoma cell line *in vitro*. Moreover, late phase tumor treatment with DOLE significantly reduced tumor volume in a syngeneic strain of mice. DOLE-treated B16 cells were blocked in the G_0/G_1 phase of the cell cycle, underwent early apoptosis and died by late necrosis. At the molecular level, the dying process started as caspase dependent, but finalized as caspase independent. In concordance, overexpression of antiapoptotic members of the Bcl-2 family, Bcl-2 and Bcl-XL, and diminished expression of their natural antagonists, Bim and p53, were observed. Despite molecular suppression of the proapoptotic process, DOLE successfully promoted cell death mainly through disruption of cell membrane integrity and late caspase-independent fragmentation of genetic material. Taken together, the results of this study indicate that DOLE possesses strong antimelanoma potential. When DOLE was applied in combination with different chemotherapeutics, various outcomes, including synergy and antagonism, were observed. This requires caution in the use of the extract as a supplementary antitumor therapeutic.

Malignant melanoma is one of the most aggressive, metastatic forms of skin cancers with increasing incidence especially in the young population. After reaching the vertical growth phase, melanoma becomes almost nonresponsive to conventional chemotherapeutic drugs.[1] Therefore, there is a rising need for the development of more efficient therapeutic approaches in the treatment of patients with melanoma. One of the most attractive ways for cancer prevention and therapy today is induction of tumor cell death and/or stimulation of a host immune response by certain phytochemicals derived from medicinal herbs and dietary plants. More than half the conventional drugs are established natural products or directly derived from them.[2–6] However, the majority of plant extracts tested so far originate from Africa, America and Eastern Asia, whereas Mediterranean flora were poorly represented in these investigations. The olive tree, *Olea europaea*, grows widely in the Mediterranean basin and parts of Asia Minor, and its medical properties have been known for a long time. Historically, the products of *O. europaea* were used as laxatives, nutritives, sedatives and tonics, as well as in specific conditions such as colic, alopecia, paralysis, rheumatic pain, sciatica and hypertension.[7] Epidemiological studies in the latter part of the 20th century revealed that the Mediterranean diet with a high level of olive products is linked to a reduced incidence of degenerative diseases, particularly coronary heart disease and cancers of the breast, skin and colon.[8] However, although the medical potential of olive fruit, including anticancer properties, is well described in the literature, data about anticancer activities of olive leaf extract are quite rare despite the historically well-known benefits of its constituents.[9–13] Components of *O. europaea* organs, such as oleuropein, tyrosol and hydroxytyrosol, have been used as antibacterial, antifungal, antiviral, anti-inflammatory and antioxidative agents.[14–18] We have recently shown the beneficial effects of dry olive leaf extract (DOLE) in two models of organ-specific autoimmunity.[19,20] Some compounds used in the treatment of infection and inflammation are efficient anticancer agents.[17,18,21,22] Accordingly, several compounds in olive leaves with antimicrobial and anti-inflammatory potential were reported to be able to induce apoptosis and differentiation of some tumor cell lines and consequently suppress their growth.[23,24] Therefore, it was of interest to evaluate the

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sensitivity of one of the most therapy-resistant cancers, melanoma, to the impressive mixture of biologically active compounds of whole olive leaf extract.

The results obtained in this study for the first time show that DOLE possesses strong antimelanoma capacity *in vitro* and *in vivo*. It was able to surmount the low responsiveness of B16 cells to classical apoptosis by induction of unconventional cell death independently of caspase activity. Besides its direct antitumor effect, DOLE intensified the action of the commonly used chemotherapeutic drugs, cisplatin and paclitaxel, but reduced the effectiveness of temozolomide and doxorubicin.

Material and Methods

Animals

Inbred C57BL/6 mice were originally purchased from Charles River Laboratories (L'Arbresle Cedex, France) and then bred and kept in our own colony at the facilities of the Institute for Biological Research "Sinisa Stankovic" (Belgrade, Serbia) under standard laboratory conditions (non-SPF) with free access to food and water. All experiments involved groups of 8 to 10 mice, matched by age (6–8 wk) and weight (20–25 g). Handling of the animals and the study protocol were in accordance with international guidelines and approved by the local Institutional Animal Care and Use Committee.

Reagents and cells

Standardized DOLE (EFLA® 943), extracted from the dried leaves of *O. europaea* L into ethanol (80% m/m), was purchased from Frutarom Switzerland (Wädenswil, Switzerland). The extract was standardized to 19.8% of oleuropein content by the manufacturer, and stability and microbiological purity were confirmed. After a patented filtration process (EFLA Hyperpure) the crude extract was dried and further analyzed for the presence of various phytochemicals as previously reported.[25] The extract was found to contain oleuropein (19.8%), total flavonoids (0.29%), including luteolin-7-*O*-glucoside (0.04%), apigenine-7-*O*-glucoside (0.07%) and quercetin (0.04%), as well as tannins (0.52%) and caffeic acid (0.02%). The DOLE preparation was water soluble, a desirable physical property for a potential drug. It was dissolved in culture medium immediately before use. Fetal calf serum (FCS), RPMI-1640, phosphate-buffered saline (PBS), dimethyl sulfoxide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), doxorubicin, paclitaxel and temozolomide were obtained from Sigma (St. Louis, MO). Annexin V-FITC (AnnV) was from Biotium (Hayward, CA). Cisplatin was a kind gift of Prof. T. Sabo (Faculty of Chemistry, University of Belgrade, Belgrade, Serbia). It was produced as described by Boreham *et al.*[26] B16 murine melanoma cell line of C57BL/6 origin was kindly provided by Prof. S. Radulovic (Institute for Oncology and Radiology of Serbia, Belgrade, Serbia). The C6 rat glioma cell line was a kind gift from Prof. Pedro Tranque (Universidad de Castilla-La Mancha, Albacete, Spain). The murine fibrosarcoma cell line, L929, was from the European Collection of Animal Cell Cultures (Salisbury, United Kingdom), while lines A375 and HCT116 were from the American Type Culture Collection (Rockville, MD). Rat insulinoma cells RINm5F (RIN) were kindly donated by Dr. Karsten Buschard (Bartholin Instituttet, Rigshospitalet, Copenhagen, Denmark). Cells were routinely maintained in HEPES-buffered RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 0.01% sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol and antibiotics (culture medium) at 37°C in a humidified atmosphere with 5% CO_2. After standard trypsinization, cells were seeded at 1×10^4/well in 96-well plates for viability tests, 2.5×10^5/well in 6-well plates for flow cytometry and real-time PCR, 5×10^2/well in 6-well plates for the cell clonogenic survival assay, and 1×10^6/25 cm^3 flask for Western blot analysis and DNA ladder.

Determination of cell viability by MTT and crystal violet assay

Mitochondrial-dependent reduction of MTT to the colored formazan product and crystal violet staining of adherent cells were used for estimation of cell viability exactly as previously described.[27,28] Cell viability was expressed as a percentage of the control value (untreated cells), which was arbitrarily set to 100%.

Cell clonogenic survival assay

Cells were treated with 0.6 mg of DOLE for 24 hr. At the end of the incubation period, cells were trypsinized, counted and 500 cells were seeded in a 6-well plate and kept for 9 days for colony formation. Culture medium was changed on Day 5. On Day 9, cell colonies were stained with 0.25 % 1,9-dimethyl-methylene blue in 50% ethanol for 45 min. Cells were washed twice in PBS, and colonies with more than 50 cells were counted.

Detection of apoptosis by AnnexinV-FITC/PI double staining, cell-cycle distribution analysis and DNA fragmentation assay

Flow cytometric analysis of phosphatidylserine staining with AnnexinV (AnnV) was used for detection of early apoptotic cells, while DNA fragmentation was explored either by staining with the DNA-binding dye PI or by DNA fragmentation assay. Autophagy was detected with acridine orange staining. Briefly, the cells were incubated in the presence or absence of DOLE for the indicated time periods, after which they were trypsinized and stained with AnnV/PI or PI, as previously described.[29] Alternatively, cells were stained with 1 μg/ml acridine orange as described earlier.[30] For detection of DNA fragmentation, the cells were treated with DOLE for 72 hr, and nucleic acid fragments were detected as previously described[31] but without the final step of treatment with RNase.

Immunoblot analysis of p-53, p^{Ser20}-p53, EndoG, Bim, Bcl-XL, Bcl-2, cyclins D1, D3 and cleaved caspase-3

B16 cells (1×10^6) were seeded in flasks (25 cm^3) and treated with the drug at the indicated time points. The cells were lysed in a solution containing 62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% wt/vol SDS, 10% glycerol, 50 mM DTT and 0.01% wt/vol bromophenol blue and separated by electrophoresis on 12% SDS-polyacrylamide gels. The samples were electrotransferred to polyvinylidene difluoride membranes at 5 mA/cm^2, using a semidry blotting system (Fastblot B43; Biorad, Goettingen, Germany). The blots were blocked with 5% wt/vol nonfat dry milk in PBS with 0.1% Tween-20 and then probed with specific antibodies to p53, p^{Ser20}-p53, cyclin D3, cyclin D1, β-actin (all from Santa Cruz Biotechnology, Santa Cruz, CA), Bim, EndoG, Bcl-XL and Bcl-2 (all from eBioscience, San Diego, CA) and active caspase-3 (R&D systems, Minneapolis) followed by incubation with secondary antibody (ECL donkey anti-rabbit HRP linked; GE Healthcare, Buckinghamshire, United Kingdom). Bands were detected by chemiluminescence (ECL; GE Healthcare).

RNA isolation and RT-PCR of caspase-3, -8 and -9 and EndoG

Total RNA was separated from B16 cells in an RNA Isolator (Metabion, Martinsried, Germany) according to the manufacturer's instructions. RNA was reverse transcribed using Moloney leukemia virus reverse transcriptase and random primers (both from Fermentas, Vilnius, Lithuania). PCR amplification of cDNA (1 μl per 20 μl of PCR reaction) was carried out in a real-time PCR machine (ABI Prism 7000; Applied Biosystems, United Kingdom) using SYBRGreen PCR master mix (Applied Biosystems) as follows: 10 minutes at 50°C for dUTP activation, 10 minutes at 95°C for initial denaturation of cDNA, followed by 40 cycles, each consisting of 15 sec of denaturation at 95°C and 60 sec for primer annealing and chain extension. Primer pairs were: caspase-3, 5′-TCT GAC TGG AAA GCC GAA ACT-3′ and 5′-AGG GAC TGG ATG AAC CAC GAC-3′; caspase-8, 5′-TCA ACT TCC TAG ACT GCA ACC G-3′ and 5′-CTC AAT TCC AAC TCG CTC ACT T-3′; caspase-9, 5′-TCC TGG TAC ATC GAG ACC TTG-3′ and 5′-AAG TCC CTT TCG CAG AAA CAG-3′; EndoG 5′-AAT GCC TGG AAC AAC CTT GAG A-3′ 5′-CAC ATA GGA CTT CCC ATC AGC C-3′and β-actin, 5′-GGA CCT GAC AGA CTA CC-3′ and 5′-GGC ATA GAG GTC TTT ACG G-3′. The expression level of each gene was calculated according to the formula $2^{-(Cti-Cta)}$ where C_{ti} is the cycle threshold of the gene of interest and C_{ta} is the cycle threshold value of β-actin. The efficiency of real-time PCR was in the optimal range of 90–110% (slope of standard curves 3.1–3.6) for all of the primer pairs used.

Induction of melanoma in C57BL/6 mice and DOLE treatment

Tumors were induced by subcutaneous injection of 2×10^5 B16 melanoma cells in the dorsal right lumbosacral region of syngeneic C57BL/6 mice. DOLE was applied at 40 mg/kg intraperitoneally (i.p.) from Day 7 after cell implantation (when tumors were not yet palpable) until the end of the experiment. Control animals were treated with diluents or cisplatin (6 mg/kg) once a week, for 4 weeks. Tumor growth was monitored daily. Urine samples were collected and biochemical parameters such as acidity, proteinuria, hematuria, ketonuria and presence of leukocytes as well as glucose, urobilinogen and bilirubin levels were monitored using semi-quantitative analysis with the "Urine strip" system (Dialab, Austria). Mice were sacrificed on Day 31. Tumor growth was determined by three-dimensional measurements of individual tumors from each mouse. Tumor volume was calculated as: $[0.52 \times a \times b^2]$, where a is the longest and b is the shortest diameter, as described previously.[30] To count the number of splenocytes, spleens were aseptically removed, gently teased through stainless steel mesh and resuspended in RPMI 1640—5% FCS. Total cell counts and viability were determined by trypan blue exclusion.

Isobologram analysis

To determine the type of interaction between DOLE and cytostatic drugs, isobolograms were made from treatments with diverse concentrations of DOLE (1.2, 0.6, 0.3 and 0.15 mg/ml) with different concentrations of cytostatic drugs as follows: cisplatin (3.25–30 μM), paclitaxel (3.75–25 μM), doxorubicin (0.125–1 μM) and temozolomide (25–200 μM). Combinations reaching 20–50% of cytotoxicity were presented as the concentration of single agent alone that produced that amount of toxicity. Analysis was made on the basis of dose-response curves of cell viability treated with DOLE alone, cytostatic drugs alone or their combination for 24 hr.

Statistical analysis

The results are presented as mean ± SD of triplicate observations from one representative of at least three experiments with similar results, unless indicated otherwise. Mann-Whitney and Student's t test were used to determine the statistical significance of differences. Values of $p < 0.05$ were considered to be statistically significant.

Results

DOLE downregulated B16 melanoma growth *in vitro* and *in vivo*

To explore the possible influence of DOLE on growth of mouse melanoma B16 cells, we treated them with different doses of the extract for 24 and 48 hr. The results obtained (Fig. 1*a*) clearly indicated that DOLE negatively regulated melanoma cell growth in a dose-dependent manner. The effect was more profound after 48 hr of incubation (Fig. 1*a*). To examine whether the extract possessed general antitumor potential, we exposed other human and rodent tumor cell lines to the same concentration range of DOLE. The extract

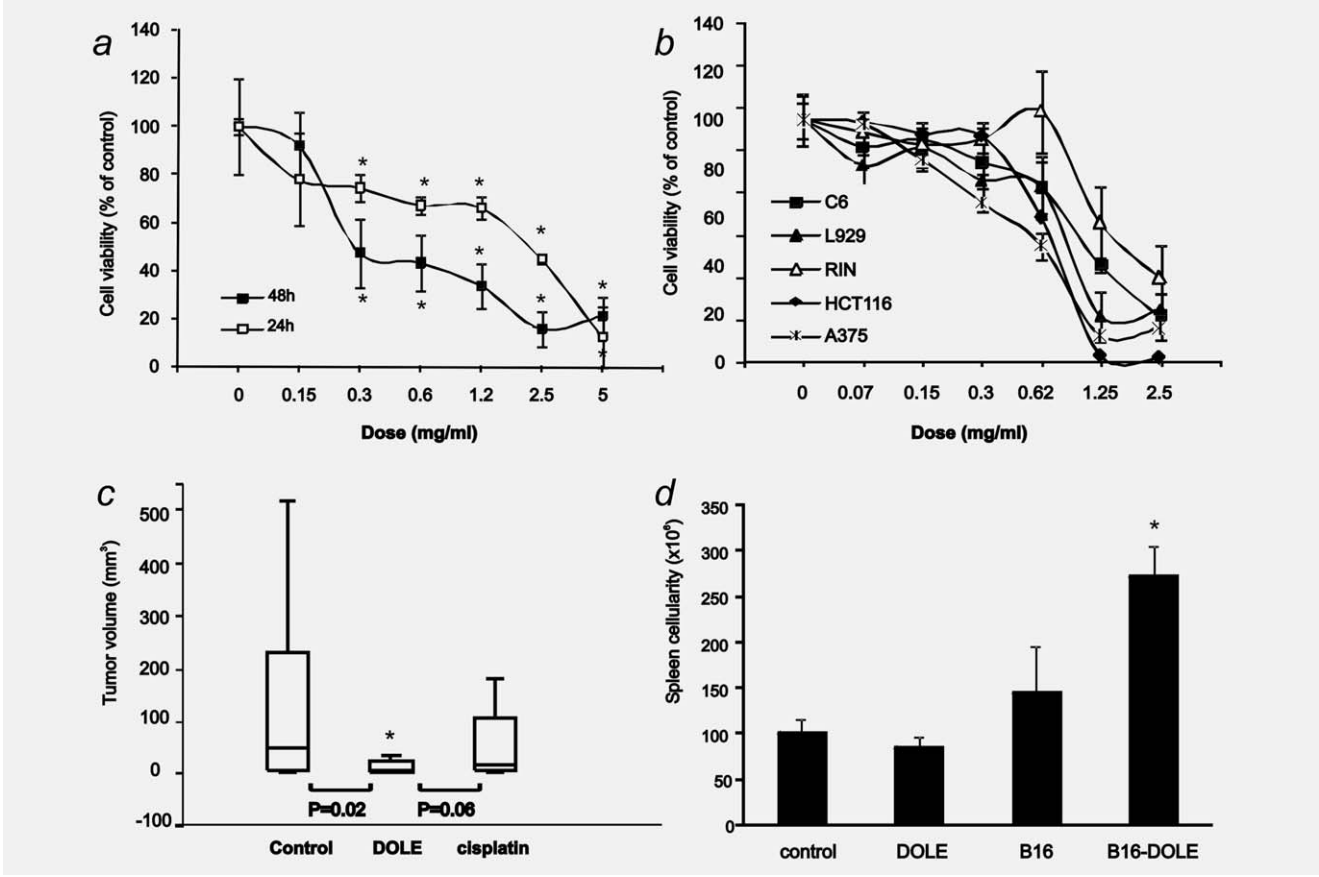

Figure 1. DOLE downregulated the growth of B16 cells *in vitro* and *in vivo*. (*a*) B16 cells or (*b*) C6, L929, RIN, HCT116 and A375 cells (1×10^4 cells/well) were treated with a range of concentrations of DOLE, after which cell viability was determined by MTT assay. The data are presented as mean ± SD from a representative of three independent experiments. *$p < 0.05$, refers to untreated cultures. (*c*) Tumors were induced by subcutaneous application of 2×10^5 B16 melanoma cells, and DOLE was applied intraperitoneally at 40 mg/kg continuously from Day 7 after cell implantation (n = 22). Cisplatin as the positive control was applied at 6 mg/kg/wk (n = 9). Tumor volume was measured on Day 31. (*d*) Tumor-induced mice were treated with PBS (B16) or with DOLE diluted in PBS (B16-DOLE) as indicated in (*c*). Normal healthy mice were treated under the same conditions with PBS (control) or with DOLE (DOLE). The number of splenocytes was counted on Day 31. Results are presented as mean ± SD from three animals per group (*$p < 0.05$ *vs* vehicle group).

nonselectively reduced cell viability of all cell lines tested (Fig. 1*b*).

The decreased viability of B16 cells observed *in vitro* was confirmed *in vivo*. B16 cells were inoculated into the dorsal right lumbosacral region of syngeneic C57BL/6 mice, and daily treatment with DOLE was started from Day 7 after implantation. The animals were killed on Day 31, and tumor volume was calculated. Application of DOLE i.p. resulted in significant reduction of solid melanomas in comparison with those in vehicle-treated controls (Fig. 1*c*). Moreover, the effectiveness of DOLE treatment was higher than the efficacy of the commonly used cytostatic drug cisplatin. In parallel, many more splenocytes (Fig. 1*d*) were obtained from tumor bearing animals treated with DOLE than from nontreated controls, indicating intensified activity of immune cells in mice that had received the extract. Treatment with DOLE did not promote any visible signs of toxicity, whereas biochemical analysis of acidity, proteinuria, hematuria and presence of leukocytes as well as urobilinogen, glucose, bilirubin and ketone levels in urine samples did not reveal noteworthy differences between control animals and animals receiving DOLE (data not shown).

DOLE triggered cell cycle arrest and subsequent death of B16 cells

To evaluate the essential mechanism responsible for decreased melanoma viability during treatment with DOLE, B16 cells were treated with the extract for 18, 24 and 48 hr, subsequently stained either with PI or Anex/PI and analyzed by cytofluorimetry. The analysis of cell cycle distribution after 18 hr of cultivation in the presence of the extract revealed radical G_0/G_1 arrest (Fig. 2*a*), which correlated with slightly upregulated expression of cyclin D1 and multiply amplified expression of cyclin D3 (Fig. 2*b*). The decreased percentage of cells in S phase was associated with morphological transformation indicating loss of proliferative potential (Fig. 2*c*).

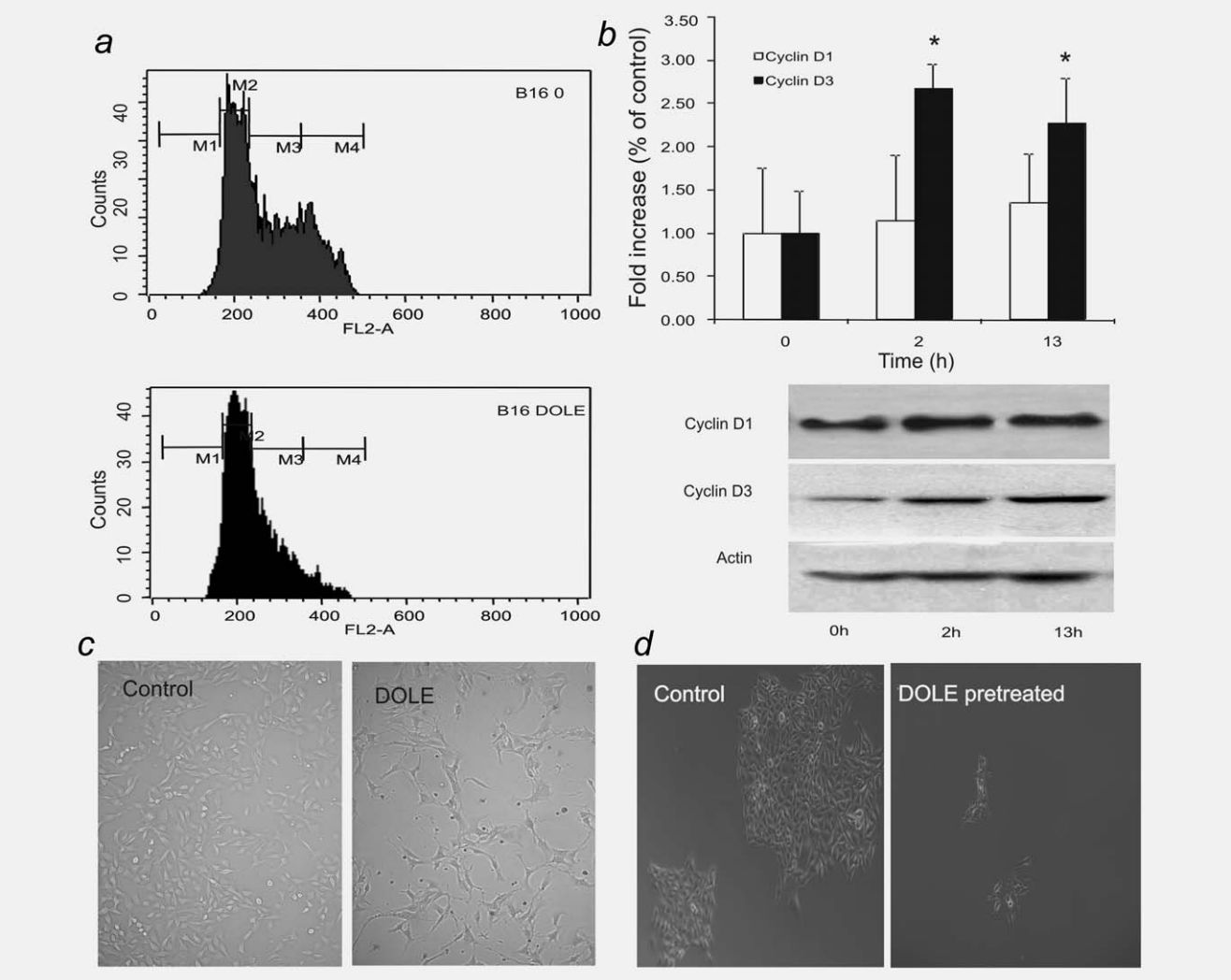

Figure 2. DOLE inhibited proliferation of B16 cells. Cells were incubated without (control) or with 1.25 mg/ml of DOLE. (*a*) Cell cycle analysis was performed by flow cytometry after 18 hr of incubation and (*b*) cyclin D1 and D3 protein expression was determined by Western blot after 2 and 13 hr of incubation. (*c*) Light microscopic evaluation of B16 cell morphology after 24 hr of the indicated treatments. (*d*) Photographs of B16 colonies at Day 9.

To clarify the effect of DOLE on cell growth, we performed a clonogenic assay. In brief, the size and number of colonies in DOLE-pretreated cultures grown for 9 days were significantly lower than in nontreated cultures [Fig. 2*d*; colonies formed per cm^2 by DOLE-treated cells and control cells were 16.0 $\pm$ 2.7 and 26.6 $\pm$ 2.0 respectively ($p < 0.05$)].

AnnV/PI staining revealed time-dependent accumulation of early apoptotic cells, marked as $AnnV^+/PI^-$ (Fig. 3*a*). After 48 hr, only 36% of the cells were viable ($AnnV^-/PI^-$), whereas almost one third of the total number of cells was $AnnV^+/PI^-$. Moreover, the large proportion of double positive ($AnnV^+/PI^+$) B16 cells indicated massive late necrosis of DOLE-treated cells at this time point (Fig. 3*a*). Microscopic observation of PI-stained cells (Fig. 3*b*) revealed that most B16 cells exposed to the extract still possessed large nuclei with only moderately condensed chromatin. The very rare presence of cells with a reduced and shrinked nucleus and highly condensed genetic material indicated that, despite massive appearance of early apoptotic markers, most cells did not reach the late stage of apoptosis and, according to the results of Anex/PI staining, probably died in a necrotic manner. To evaluate the eventual contribution of autophagic cell death in the antimelanoma action of DOLE, we analyzed the presence of autophagosomes in the cytoplasm of DOLE-treated cells. The absence of autophagic vesicles in their cytoplasm indicated the irrelevance of autophagy either as a death-promoting or a cytoprotective mechanism (Fig. 3*c*). In addition, fragmentation of genomic DNA isolated from DOLE-treated cells was not detectable before 72 hr of treatment, indicating that nuclear destruction came very late as the final stage of the triggered death process (Fig. 3*d*).

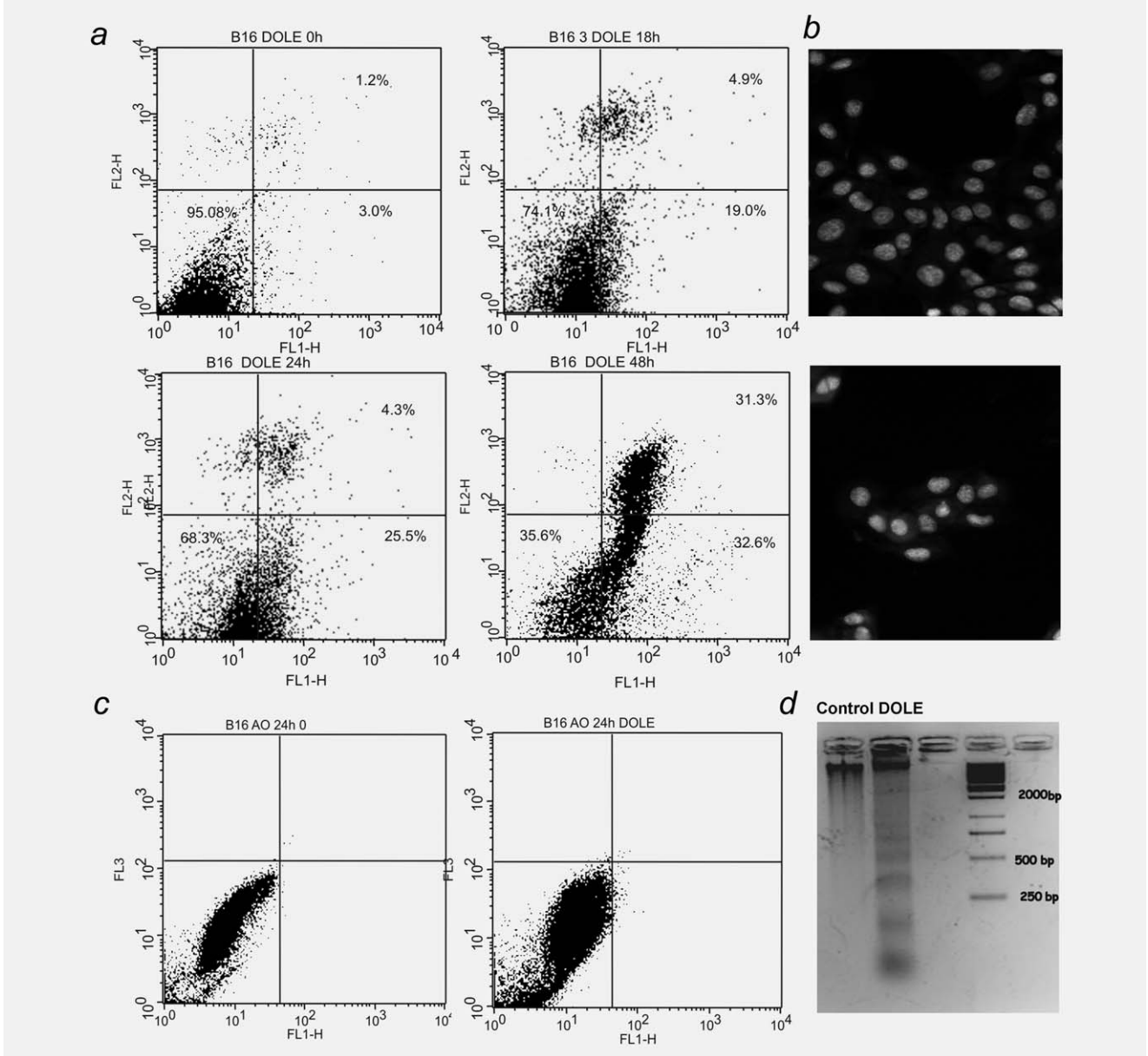

Figure 3. DOLE induced primary apoptosis and secondary necrosis in B16 cells. (*a*) Cells were incubated without (control) or with 1.25 mg/ml of DOLE for 18, 24 and 48 hr and then stained by AnnV/PI. (*b*) PI-stained cells were evaluated under the microscope after 48 hr. (*c*) Staining with acridin-orange was done after 24 hr. (*d*) DNA fragmentation assay was performed after 72 hr of cultivation.

DOLE induced switch from caspase-dependent to caspase-independent death by targeting the balance between pro- and antiapoptotic mediators in B16 cells

To explain the basic mechanism of the dying process promoted by DOLE, we analyzed the expression of caspase-3, a crucial executioner in apoptosis. After 2 and 13 hr of incubation in the presence of the extract, immunoblot analysis revealed transient, time-limited upregulation of the active form of caspase-3, followed by a significant decrease of expression (Fig. 4*a*, left panel). Evaluation of caspase-3, -8 and -9 genes expression disclosed remarkable decreases in their transcription (Fig. 4*a*, right panel). To explore possible causes of breakdown and subsequent inhibition of caspase-3, first, we analyzed the expression of the cell protective Bcl-2 family members, Bcl-2 and Bcl-XL, key proteins for the development of resistance to apoptosis and their natural inhibitors, Bim and p53.[32–37] The results obtained revealed moderate stimulation of Bcl-2 expression and powerful upregulation of Bcl-XL expression 2 hr after addition of the extract, with a slight tendency to decrease in the next 11 hr (Fig. 4*b*). In parallel, p53 and particularly Bim were considerably reduced in the same time interval (Fig. 4*c*). Failure of caspase protein expression in cells exposed to DOLE could be related to inability of Bim and p53 to block Bcl-2 action.

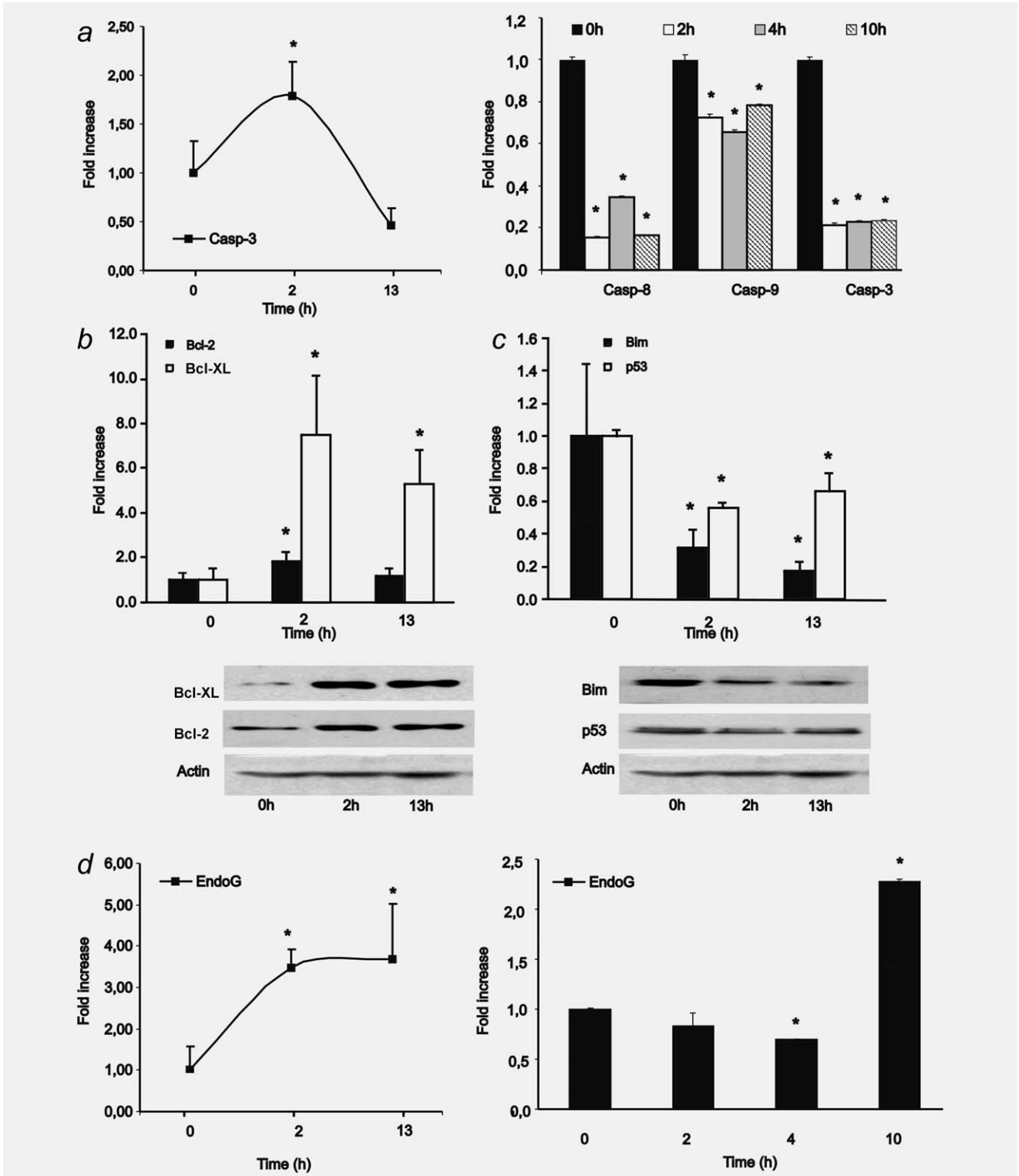

Figure 4. DOLE oppositely regulated caspase and EndoG expression. Cells were incubated without (control) or with 1.25 mg/ml of DOLE, and the Western blots were analyzed densitometrically for active caspase-3 (*a*, left panel), Bcl-2, Bcl-XL (*b*), Bim and p53 (*c*) and EndoG protein expression (*d*). The results are presented as fold increase relative to control (mean ± SD for two independent experiments). *$p < 0.05$, refers to untreated cultures. RT-PCR for caspase-3, -8 and -9 (*a*, right panel) and EndoG (*d*, right panel) was performed at the indicated time points. The data are presented as mean ± SD for two independent experiments. *$p < 0.05$, refers to untreated cultures.

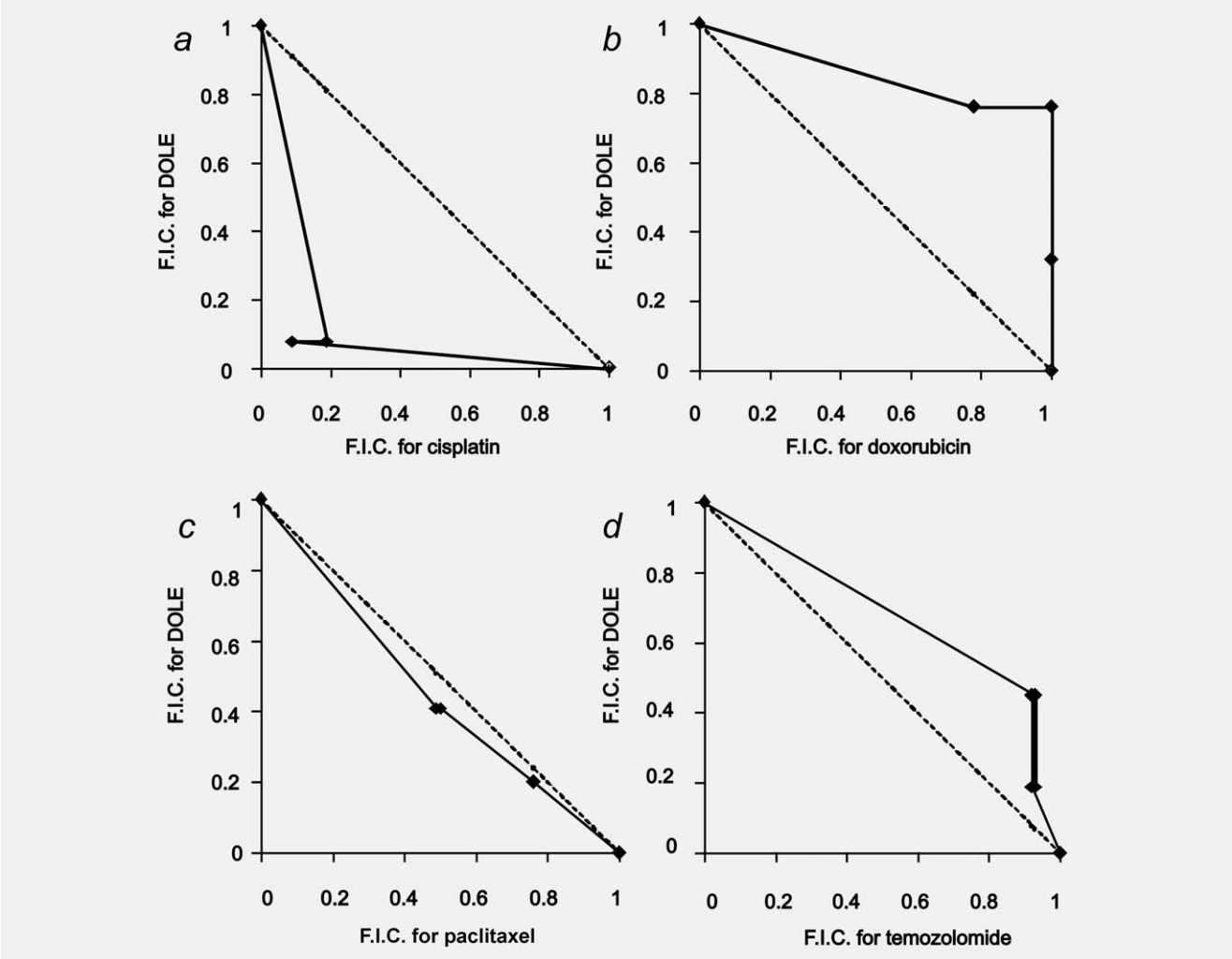

Figure 5. DOLE differently affected the responsiveness of B16 cells to cytostatic drugs. Cells (1×10^4 cells/well) were treated with various concentrations of cisplatin (*a*), paclitaxel (*b*), doxorubicin (*c*) or temozolomide (*d*) in the presence of 0.15–0.6 mg/ml of DOLE. After 24 hr, cell viability was determined by CV assay, and Isobologram curves from a representative of three independent experiments are presented. (Fraction inhibitory concentration: concentration of each agent in combination/concentration of each agent alone, F.I.C. < 1 is considered synergistic; F.I.C. > 1 is considered antagonistic).

Despite caspase inhibition, intensive DNA fragmentation was detected, as shown by the DNA ladder (Fig. 3*d*). At the same time, protein expression of caspase-independent endonuclease, EndoG, rapidly increased to reach thrice the values found in untreated cultures after 13 hr of incubation with DOLE (Fig. 4*d*, left panel). Accordingly, *EndoG* gene expression was intensified after 10 hr of the treatment (Fig. 4*d*, right panel). Upregulated expression of *EndoG* corroborated with the low length fragmentation of DNA obtained under the influence of DOLE (Fig. 3*d*).

DOLE modulated the effectiveness of cytostatic drugs

As plant extracts have often been used as supplements to chemotherapies, we next evaluated potential interactions between DOLE and the most commonly used cytostatic drugs. Cells were treated with a wide range of doses of cisplatin, doxorubicin, paclitaxel or temozolomide in the absence or presence of low-toxic concentrations of DOLE. The MTT assay was performed after 24 hr of incubation, and drug interactions were evaluated by isobologram analysis. The results presented in Figure 5 clearly show that addition of DOLE differentially affected the toxicity of the applied drugs against B16 cells. Responsiveness to cisplatin and paclitaxel was significantly intensified in the presence of the extract. Isobologram curves indicated that addition of DOLE synergized with the toxicity of these drugs (Figs. 5*a* and 5*b*). On the contrary, parallel treatment with DOLE antagonized the action of doxorubicin or temozolomide in B16 cell cultures (Figs. 5*c* and 5*d*). Besides their direct antimelanoma potential, these data suggest that constituents of DOLE could either amplify or reduce the effectiveness of chemotherapy depending on the type of cytostatic drug.

Discussion

Olive leaf extracts are primarily consumed as natural inhibitors of replication of many pathogens. Their potent bioactivity and relatively low toxicity have rendered them useful ingredients in complementary alternative medical and nutritional supplements. Here, we provide the first evidence of antimelanoma activity *in vitro* and *in vivo*. These findings are in line with the recently demonstrated anticancer effects of olive tree-derived phytochemicals.[18,22–24,38] The extract used here contained a high percentage of phenols, including 0.29% of flavonoids and 0.52% of tannins. Oleuropein, caffeic acid, luteolin, luteolin-7-*O*-glycoside, apigenin-7-*O*-glycoside, quercetin and chryseriol were recognized and quantified.[25] All of them are already described active molecules able to affect cell physiology in multiple ways. Oleuropein, the major constituent of DOLE (nearly 20% of the extract) has been shown to inhibit growth, motility and invasiveness of cancer cells. On the other hand, most normal cells exposed to oleuropein showed only reversible changes in morphology without significant alteration in cell viability.[18,22] The flavonoids, luteolin, quercetin, chryseriol and apigenin, not only prevent carcinogenesis but also inhibit angiogenesis, induce apoptosis, reduce tumor growth *in vivo*, cooperate with cytostatic drugs or sensitize tumor cells to the cytotoxic effects of some chemotherapeutics.[39] Possible mechanisms involved in their anticancer activities at the intracellular level are modulation of ROS and RNS production, inhibition of topoisomerases I and II, reduction of NF-kappaB and AP-1 activity, stabilization of p53 and inhibition of p38, PI3K, STAT3, IGF1R and HER2.[39] In view of the fact that the extract is a mixture of biologically active compounds that directly or indirectly influence tumor growth, the net effect is defined by their interplay. Because purification of herbal extracts to single compounds usually results in loss of biological activity,[40] we may also suppose that the association of various substances in DOLE, with their complex interactions and different cellular and molecular targets might be responsible for the impressive antimelanoma activity of the extract documented here. Thus, DOLE possesses strong capacity to downregulate growth of melanoma *in vitro* and *in vivo*. The prevention of tumor growth was exerted through diverse mechanisms, including cell cycle arrest, lost of proliferative and, therefore, clonogenic properties and subsequent induction of tumor cell death, which started in a caspase-dependent but finished in a caspase-independent manner. Decreased expression of the active form of caspase-3 correlated with upregulation of antiapoptotic Bcl-XL and suppressed expression of its natural antagonists, Bim and p53. Bim was previously described as a limiting factor in p53-mediated apoptosis, the deficiency of which was able to abrogate apoptosis.[33,34] This proapoptotic protein binds to Bcl-2 and Bcl-XL preventing their defensive function and, in parallel, liberates proapoptotic Bax.[35,41] The other relevant protein, p53, besides numerous roles in regulation of the apoptotic process at the transcriptional level, actively modulates apoptosis by direct interaction with antiapoptotic members of the Bcl-2 family.[42] The outcome of this high affinity interaction is similar to that observed with Bim and resulted in breakdown of the Bcl-2-mediated protective signal.[43] A continuous and intensive Bim decline, as well as the temporary p53 decrease triggered by DOLE, synchronized with Bcl-XL amplification, was probably responsible for caspase-3 inactivation, inhibited transcription of caspases-3, -8 and -9 and subsequent deviation from the typical apoptotic process with a shift to necrosis. Inhibition of caspase genes could be the consequence of multiple changes in cell physiology modified by constituents of DOLE (ROS and RNS production and NFkB activity).[11,39,44,45] In addition, these intracellular events were in complete concordance with results obtained by AnnV/PI staining and morphological evaluation of vital or PI-stained cells after 48 hr incubation with DOLE. The observed necrosis could be an important stimulus for both nonspecific and adaptive immune responses against tumor cells in contrast to the low immunogenic apoptosis, which usually ends with ingestion of cell residues by nearby cells without local tissue destruction and inflammation. Fragmented DNA as the final stage of nuclear dismantling was not detected before 72 hr. In the absence of active caspase-3, the stimulated transcription and translation of EndoG pointed to its possible involvement in DNA dismantling. This specific caspase-independent endonuclease, located in the inner membrane of mitochondria, is a unique mammalian DNase capable of making single-strand breaks in DNA.[46,47] The fast upregulation of mature EndoG expression observed during the first 2 hr of cultivation with DOLE unsynchronized with changes in intensity of transcription could be related to translation of preexisting mRNA,[47] rather than a consequence of *de novo* gene expression. Low length fragments generated by intranucleosomal cleavage of genetic material induced by DOLE treatment also indicated that EndoG could be relevant for finalization of the triggered dying process. It is possible that decreased levels of the active form of caspase-3, probably mediated by Bcl-2 overexpression, represented a defensive reaction of B16 cells to proapoptotic stimuli. Indeed, unresponsiveness of these cells to induction of apoptosis with different mediators has already been described.[48] Therefore, it was of interest to evaluate the possible interaction of DOLE with conventional therapies. Treatment with low toxic doses of DOLE significantly intensified B16 cell responsiveness to cisplatin and paclitaxel *in vitro*. The mechanism behind this effect could be related to cyclin D3 expression. Despite the many prosurvival functions of cyclin D3 described in the literature, increased activity of this molecule was directly responsible for sensitization of HeLa cells to immune responses against the tumor and was connected with apoptotic cell death induced by cisplatin.[49] In contrast, combination of DOLE with other cytostatic drugs, such as doxorubicin or temozolomide, decreased their effectiveness indicating a negative outcome of these interactions. Many factors can determine the result of combined treatments, such as the essential mechanism of drug

action, operative intracellular signalization and cell specificity, together with many others. It is known that some DOLE constituents possess strong antioxidant activity and are able to neutralize cell toxicity triggered by hyperproduction of free radicals.[14–18] On the other hand, the primary goal of herbal supplementation in cancer therapy is reconstitution of immune system functions. The observed increase of spleen cell number in tumor bearing mice on DOLE treatment indicated enhanced immunoreactivity. Immunostimulating agents can strongly boost effector and memory function of tumor killing NK cells, macrophages, $CD4^+$ and $CD8^+$ T cells.[50] Taken together, uncontrolled utilization of conventional drugs with alternative medicine, such as dietary supplements, might be a double-edged sword. Having in mind that melanomas are tumors with high metastatic potential, mainly resistant to chemo- and radiotherapy, the potent antitumor activity of DOLE observed both *in vitro* and *in vivo*, as well as its capacity to increase the effectiveness of chemotherapy, along with the observed safety profile, suggest its potential usefulness in antimelanoma therapy. The encouraging results presented in this article impose further preclinical investigation of the antimelanoma properties of DOLE to evaluate the possible translation of these findings to the clinical setting.

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