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ORIGINAL ARTICLE

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Inhibition of HUVEC tubulogenesis by hederacolchiside-A1 is associated with plasma membrane cholesterol sequestration and activation of the Ha-Ras/MEK/ERK cascade

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Abstract *Purpose*: Neoangiogenesis is critical to cancer proliferation and metastasis and constitutes an attractive target for cancer therapy. It has previously been demonstrated that hederacolchiside-A1 (HCol-A1), a triterpenoid saponin from *Hedera colchica* Koch, has antimelanoma potential. The goal of this study was to evaluate, in vitro, if in addition to its tumoricidal effect on melanoma cells, HCol-A1 might affect endothelial cell network formation. *Methods*: We investigated whether HCol-A1 affects matrigel-induced tubulogenesis and inhibits the viability (WST-1 assay) of human umbilical vein endothelial cells (HUVECs). To provide structure-activity relationships (SAR), studies were conducted on HCol-A1, oleanolic acid and hederacolchiside A (HCol-A), a triterpenoid saponin which possess the same sugar sequence as Hcol-A1. Plasma membrane cholesterol sequestration was studied by labelling with [^{3}H]cholesterol and assayed with HCol-A1-cholesterol complexes. HCol-A1 signalling was investigated using immunoassays. *Results*: In contrast to HCol-A and oleanolic acid, HCol-A1 inhibited matrigel-induced angiogenesis at micromolar concentration. Plasma membrane cholesterol sequestration was found to be critical for this activity. Activation of the *Ras*/MEK/ERK cascade appears to be one of the mechanisms by which Hcol-A1 affects HUVEC network formation. The predominant activation of the Ha-*Ras* isoform, which decreases HUVEC-tolerance to apoptosis, might contribute to the high susceptibility of this cell line to HCol-A1. *Conclusion*: Since cholesterol sequestration affects cell confluence-dependent remodelling of endothelial membranes and vascular endothelial growth factor receptor-2 activity, these results raise the possibility that Hcol-A1 might slow-down cancer proliferation and metastasis in vivo by inhibiting critical aspects of neoangiogenesis. Further in vivo studies are needed to verify this hypothesis.

Keywords Cancer · Angiogenesis · Triterpene saponin · Hederacolchiside-A1 · Cholesterol · ERK · Ras

Abbreviations CC: Column chromatography · DMEM: Dulbecco's modified Eagle's medium · EGCg: Epigallocatechin gallate · FBS: Fetal bovine serum · HCol-A1: Hederacolchiside A1 · HCol-A: Hederacolchiside A · Ha-*Ras*: Harvey isoform of *Ras* · Ki-*Ras*: Kirsten isoform of *Ras* · mAb: Monoclonal antibody · pAb: Polyclonal antibody · PBS: Phosphate-buffered saline · PVDF: Polyvinylidene difluoride · RP-HPLC: Reverse-phase high-pressure liquid chromatography · ROS: Reactive oxygen species · SAK: Extracellular signal-regulated kinase · VEGFR-2: Vascular endothelial growth factor receptor-2

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Introduction

To proliferate, tumours need to both partially inhibit pathways leading to apoptosis and to initiate their own blood supply via the development of new blood vessels without which they cannot growth beyond a size of 1–2 mm and cannot metastasize [1]. The recognition that tumour-induced neovascularization is critical to aggressive tumour proliferation and metastasis has led

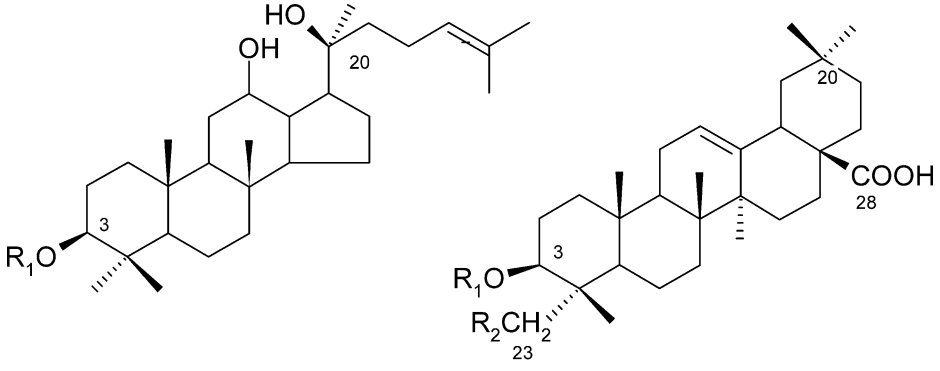
to the development of new strategies for cancer therapy and the usefulness of this approach is now well established (for review, see references 2 and 3). As a consequence, much effort has been directed towards identifying agents aimed at preventing critical aspects of angiogenesis such as endothelial cell proliferation, migration, adhesion and reorganization.

Saponins are steroidal or triterpenoid glycosides, common in a large number of plants and plant products that are important in human and animal nutrition. It has recently been found that ginsenosides Rg-3 and Rh-2, two purified ginseng saponins with a dammarane skeleton (see structures in Fig. 1), significantly decrease the incidence of cancer metastasis in vitro and in vivo [4–8]. The mechanism of their antimetastatic effect has been associated with inhibition of tumour-associated angiogenesis and also, for ginsenoside Rg-3, with inhibition of tumour cell invasion [4–8]. Ginsenoside Rg-3 is already marketed in China under the name of "Rg-3 shenyi Jiaonang" (2×10 mg of 95% Rg-3 per capsule, twice a day before a meal) to prevent adhesion, invasion and metastasis of tumour cells [9].

Fig. 1 Structures of the different hederasaponins tested in the present study as compared to structures of ginsenosides Rg-3 and Rh-2 isolated from red ginseng. Ginsenosides Rg-3 and Rh-2 are steroid (tetracyclic) saponins whereas HCol-A1 and HCol-A are triterpene (pentacyclic) saponins. The aglycone (sapogenin) of Rg-3 and Rh-2 is 20 (*S*)-protopanaxadiol. The aglycones of HCol-A1 and HCol-A are oleanolic acid (R1 = H) and hederagenin (R1 = OH), respectively. All these drugs are monodesmosides, i.e., saponins exhibiting one sugar chain linked at C3–(OH). The sugar chain of HCol-A1 and HCol-A is Rha1–2[Glc1–4]Ara, where Ara (L-arabinose), Rha (L-rhamnose) or Glc (D-glucose), are all in a pyranosyl form. Rg-3 and Rh-2 only contain Glc. *Note:* white ginseng prepared by drying after peeling roots and rhizomes of *Panax ginseng* C.A. Meyer (Araliaceae) contains more than 25 damnarane-type sapogenins. The most usual ginsenosides (Rb-1, Rb-2, Rc and Rd) are esterified bidesmosides characterized by the presence at C3–(OH) of a complementary malonyl group at the 6-position of the second glucosyl moiety, and at C20–(OH) of a second sugar chain which can be either Glc^{6}–Glc (Rb-1) or Glc^{6}-Ara (Rb-2, Rc; Ara is in a pyranosyl form in Rb-2 and in a furanosyl form in Rc) or Glc (Rd). Red ginseng is prepared by steaming and drying roots and rhizomes. During this step, the malonyl group is released and the sugar moiety at C20-(OH) is partly lost, leading to the formation of Rh-2 and Rg-3 which appear to be more active than the natural ginsenosides Rb-1, Rb-2, Rc and Rd against human cancer [7]

Experimental and epidemiological experiments have suggested that ginseng saponins might also have a cancer-preventive effect [10]. Although various data have demonstrated that triterpenoid saponins inhibit tumour cell proliferation in vivo and in vitro (for review, see reference 11), the capacity of these pentacyclin saponins to affect tumor angiogenesis remains to be explored. In addition, little is known about the molecular mechanisms by which these saponins inhibit tumour proliferation. We have previously reported that a new triterpenoid saponin with an olean 12-ene skeleton isolated from the ivy species, *Hedera colchica* K. Koch, Hederacolchiside A1 [HCol-A1, 3-*O*-{αL-Rha (1-2)-[βD-Glcp (1-4)]-αL-Arap}-oleanolic acid; Fig. 1], blocks the proliferation (IC_{50} 4.5–12 μ*M*) of a panel of human carcinoma cell lines with a preferential toxicity and a positive cytotoxic index (CI 1.8) on a pigmented human malignant melanoma cell line [12]. The high susceptibility of melanoma cells to the drug was proved to be inversely proportional to the melanin content of each cell line [13]. It was suggested that in addition to early membrane-damaging effects identified as the main cause of toxicity, the capacity of HCol-A1 to bind melanin, might contribute to its high toxicity on pigmented cell lines [13].

Malignant melanoma represents the most common form of fatal skin cancer. Its incidence is increasing at a rate of 5% per year. During the initial stage of development, melanoma can be usually cured by surgery, but once the metastatic phase develops, it is almost always fatal. The overall survival for patients with metastatic melanoma ranges from 4.7 to 11 months, with a median survival of 8.5 months [14]. Therefore, agents that inhibit or limit the proliferation of melanoma metastases have the potential to be of considerable therapeutic value. The physiological activity of triterpenoid saponins is usually associated with their ability to complex sterols [15], especially plasma membrane cholesterol [16]. Together with sphingomyelin, cholesterol is an essential constituent of plasma membrane rafts or caveolae that are sites where signalling molecules are concentrated and which spatially organizes signal transduction at the cell surface [17]. It is now well established that cholesterol is



critically important for this process and that agents that bind and/or extract raft lipids (cholesterol and sphingolipids) alter the localization, the trafficking and the function of the raft-associated proteins [18, 19].

We therefore addressed the possibility that HCol-A1 might inhibit angiogenesis by inducing cholesterol depletion and, therefore, have the potential to limit the proliferation of melanoma metastases. The present study showed that (1) Hcol-A1 affects HUVEC network formation in the matrigel assay and (2) cholesterol sequestration and Ras/MEK/ERK signalling are involved in the process. Assays were conducted versus oleanolic acid, the aglycone of HCol-A1, and versus HCol-A (another modesmoside from *H. colchica*, with the same sugar sequence as HCol-A1 from which it differs only by the replacement of a proton by a hydroxyl at C24 of the sapogenin) to provide structure-activity information.

Material and methods

Chemicals and plant materials

Low glucose DMEM, RPMI 1640 and other medium components were purchased from Gibco BRL (Paisley, UK). WST-1 reagent and complete TM protease inhibitors were from Roche Molecular Biochemicals (Laval, Quebec, Canada, and Meylan, France). Inhibitor PD98059 (MEK1-specific inhibitor) was from Calbiochem (La Jolla, Calif.). Nonidet P-40 (NP-40) and Brij 96 were from Fluka (Oakville, Ontario, Canada). Rabbit monoclonal antibody (mAb) phospho-ERK1/2 IgG that recognizes phosphorylated active form of ERK1/2 (anti-phospho Thr^{202}/Tyr^{204}, pERK IgG), and mAb against p44/42, phospho-p38 and phospho-JNK were New England Biolabs (Boston, Mass.). pAb against total ERK1/2, polyclonal anti-pan-Ras and mAb against Ha-Ras and Ki-Ras were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.); Anti-p17 mAb was from PharMingen (San Diego, Calif.). Anti-cytochrome *c* and anti-pro caspase-8 Ab were from Biomol Research Laboratories (Plymouth Meeting, Pa.). Anti-mouse and anti-rabbit IgG horseradish peroxidase-linked whole Ab were purchased from Jackson Immuno-Research Laboratories (West Grove, Pa.). Protein A-sepharose, protein G-sepharose and enhanced chemiluminescence (ECL) reagent were from Amersham Pharmacia Biotech (Oakville, Ontario, Canada). [^{3}H]Cholesterol was from NEN Life Science Products. All other reagents were from Sigma Aldrich (Oakville, Ontario, Canada, or L'Isle d'Abeau, France).

Saponin isolation HCol-A1 and HCol-A were isolated from dried leaves of *H. colchica* (Araliaceae) as described previously [7, 12] with a complementary HPLC step. The structure was unambiguously identified by NMR analysis according to the methods described by Mshvildadze et al. [20] for Hcol-A1 and Hcol-A. In brief, separation involved extraction from dried leaves with 80% ethanol, partition against chloroform, evaporation of the water phase and repeated column chromatography of the residue on silica gel-L-40/100 with chloroform/methanol/water (26:14:3). The resulting compounds were submitted to a 90-min alkaline hydrolysis with 5% aqueous KOH at 100°C, then extracted with butanol and purified by column chromatography on Si-60 gels. Pure compounds (up 95% purity for HCol-A1 and up 87% for HCol-A) were obtained by RP-HPLC purification on μ-Bondapack C-18 10 μm, 300×3.9 mm (Waters) with $CH_3CN/H_2O/H_3PO_4$ (50:49.89:0.11) at 1 ml/min. Oleanolic acid was a gift of Prof. G. Balansard (Faculté de Pharmacie, Marseille, France).

Cell culture

HUVECs (Clonetics Corporation, San Diego, Calif.) were cultured in low-glucose DMEM containing 10% heat-inactivated FBS with antibiotics and cultured at 37°C under a humidified atmosphere containing 5% CO_2. In some experiments, HUVECs were maintained in RPMI 1640 with 10% heat-inactivated FBS and 2 mmol glutamine and were used at passage 5–10.

Matrigel endothelial cell tube formation assay

Matrigel (12.5 mg/ml) was thawed at 40°C, and 50 μl was quickly added to each well of a 96-well plate and allowed to solidify for 30 min at 37°C. Once solid, the wells were incubated for 30 min with HUVECs (30,000 cells/well). After cell adhesion, the medium was removed and replaced by fresh medium supplemented with the indicated concentrations of HCol-A1, HCol-A, oleanolic acid or solvent mixture (control), and incubated at 37°C for 18 h. Tube formation was visualized with an inverted Zeiss Axiovert S100 microscope at a magnification of ×50, and images were digitalized using a computer-assisted imaging system equipped with a QImaging Retiga 1300 camera. The length of the capillary network was quantified using Northern Eclipse software (Empix, Mississauga, Ontario, Canada).

HUVEC viability assay

HUVECs were plated in 96-well plates at a 5000 cells/well in 200 μl complete medium and incubated at 37°C under an atmosphere containing 5% CO_2 for 24 h. The next day, the medium was removed and replaced by 100 μl fresh medium containing 0.5% serum, no endothelial factors and the specified concentrations of saponin. Cell viability was determined by assaying the residual mitochondrial activity of treated cells at 6, 24 and 48 h with the highly sensitive WST-1 assay. Briefly, 10 μl of WST-1 reagent (a tetrazolium salt) was added to each well and the soluble formazan dye produced by

metabolically active cells was monitored for 1 h at 37°C on a SpectraMax Plus plate reader (Molecular Devices). When needed, cells were preincubated overnight with PD98059 (40 μmol).

Incorporation of [^{3}H]cholesterol

Cells were cultured overnight in medium supplemented with [^{3}H]cholesterol (NEN Life Science Products) at 12.5 μCi/ml as described by Corvera et al. [21]. The cells were then rinsed three times with unlabelled medium, returned to the incubator for 1 h more, were treated or not with cyclodextrin (6%) or Hcol-A1 for the indicated time, and were harvested as described above. Radiolabel content was quantitated by scintillation counting of postnuclear supernatants, prepared as described above, and normalized for protein.

Preparation of Hcol-A1-cholesterol complexes

Complexes were prepared by a modification of the procedure described by Klein et al. [22] for the preparation of β-cyclodextrin–cholesterol complexes. Briefly, 10 μl of a 6% solution of cholesterol in isopropyl alcohol was added to 250 μl of a 1% solution of Hcol-A1 in the same solvent. After dispersal of the cholesterol, the solution was maintained at 37°C with stirring and directly used, or lyophilized and reconstituted in DMSO before use.

Immunoblotting assays

HUVECs were cultured to 70% confluence, washed twice with PBS then lysed on ice in NP-40/Brij buffer (50 m *M* Tris-HCl, pH 7.5, 1% Nonidet P-40 9NP40), 1% Brij 96, 1 m *M* Na_3VO_4, 10 m *M* β-glycerophosphate, 10 m *M* NaF, 2 m *M* EDTA, and a cocktail of proteases inhibitors) to solubilize raft- and nonraft-associated proteins. The resulting lysates were clarified by centrifugation at 10,000 *g* for 10 min. Samples containing identical amounts of proteins (20, 50 or 100 μg) were boiled for 4 min and subjected to 12.5% SDS-PAGE. For immunodetection of pERK, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes, incubated for 60 min with Tris-buffered saline/Tween-20 buffer (TBST; 147 m *M* NaCl, 20 m *M* Tris-HCl, pH 7.5, with 0.1% Tween 20) containing 5% nonfat dried milk (NFDM), then overnight at 4°C with primary antibody. Immunodetections were performed using primary antibodies directed against the phosphorylated (active) forms of ERK, at concentrations recommended by the supplier. Immunoreactive bands were revealed after 1 h incubation with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies, and the signals were visualized with an ECL detection system and exposure to X-ray film. After stripping, membranes were reprobed with polyclonal antibodies against the total (phosphorylated and unphosphorylated) ERK1/2 or against ERK-2.

For pro-caspase-3, and cytochrome *c*, immunocomplexes resulting from overnight incubation at 4°C with specific antibodies were collected by incubating lysates with 25 μl of protein A-sepharose beads (50% suspension) for 2 h at 4°C. Immunoprecipitates were washed three times with 50 mmol Tris-HCl buffer, pH 7.5, containing 1 m *M* EDTA, 0.5% NP40 and once with PBS, pH 7.4, containing 1 m *M* Na_3VO_4, centrifuged and resuspended in 125 m *M* Tris-HCl buffer, pH 6.8, containing 20% glycerol, 5% SDS, 10% β-mercaptoethanol and 0.00125% bromophenol blue SDS sample buffer, boiled for 4 min, and analyzed by Western blotting as described above. β-Actin was used as a control of the loaded amount of protein. For immunodetection of Ki-*ras* and Ha-*ras*, immunocomplexes resulting from incubation overnight at 4°C with a polyclonal anti-pan Ras antibody were collected by a 1-h incubation at 4°C with 25 μl of protein G beads. Immunoprecipitates were treated as described above. Blots were probed with a specific monoclonal antibody against Ha-*Ras* and ki-*Ras*.

Results and discussion

Dose-dependent inhibition of matrigel-induced formation of endothelial cell capillary-like structures by HCol-A1

The matrigel-induced tubulogenesis assay is widely used to identify antiangiogenic drug candidates [23]. In this assay, the cell reorganization, named tubulogenesis, occurred in three steps. In the first (about 2 h), HUVECs adhered to the matrix and were homogeneously distributed. Areas free of cells appeared 6–8 h later. Finally, between 10 and 24 h after seeding, thin tubules which resembled capillaries were formed at the junction of the cell free areas. This latter effect was not affected by a 18-h continuous contact with HCol-A or oleanolic acid (Fig. 2a). In contrast, it was strongly decreased when HCol-A1 was added to the culture medium at concentrations up to 2.5 μg/ml, and was completely abolished at 3 μg/ml (Fig. 2b). At the latter concentration, HCol-A1 induced a higher inhibition of capillary-like structures than epigallocatechin gallate (EGCg, 10 μg/ml), a vascular endothelial growth factor receptor-2 (VEGFR-2) inhibitor from green tea [24], which was used as a positive control (Fig. 2c). Although these findings suggest a threshold rather than a dose-dependent effect, they unambiguously demonstrate that in vitro, HCol-A1 affects the formation of new vessels at the micromolar level. Interestingly, the endothelial cell network was completely inhibited at IC_{50} value for inhibition of melanoma cell proliferation (3.75–4.5 μg/ml). To the best of our knowledge, these findings provide the first evidence that in addition to their tumoricidal effect, cer-

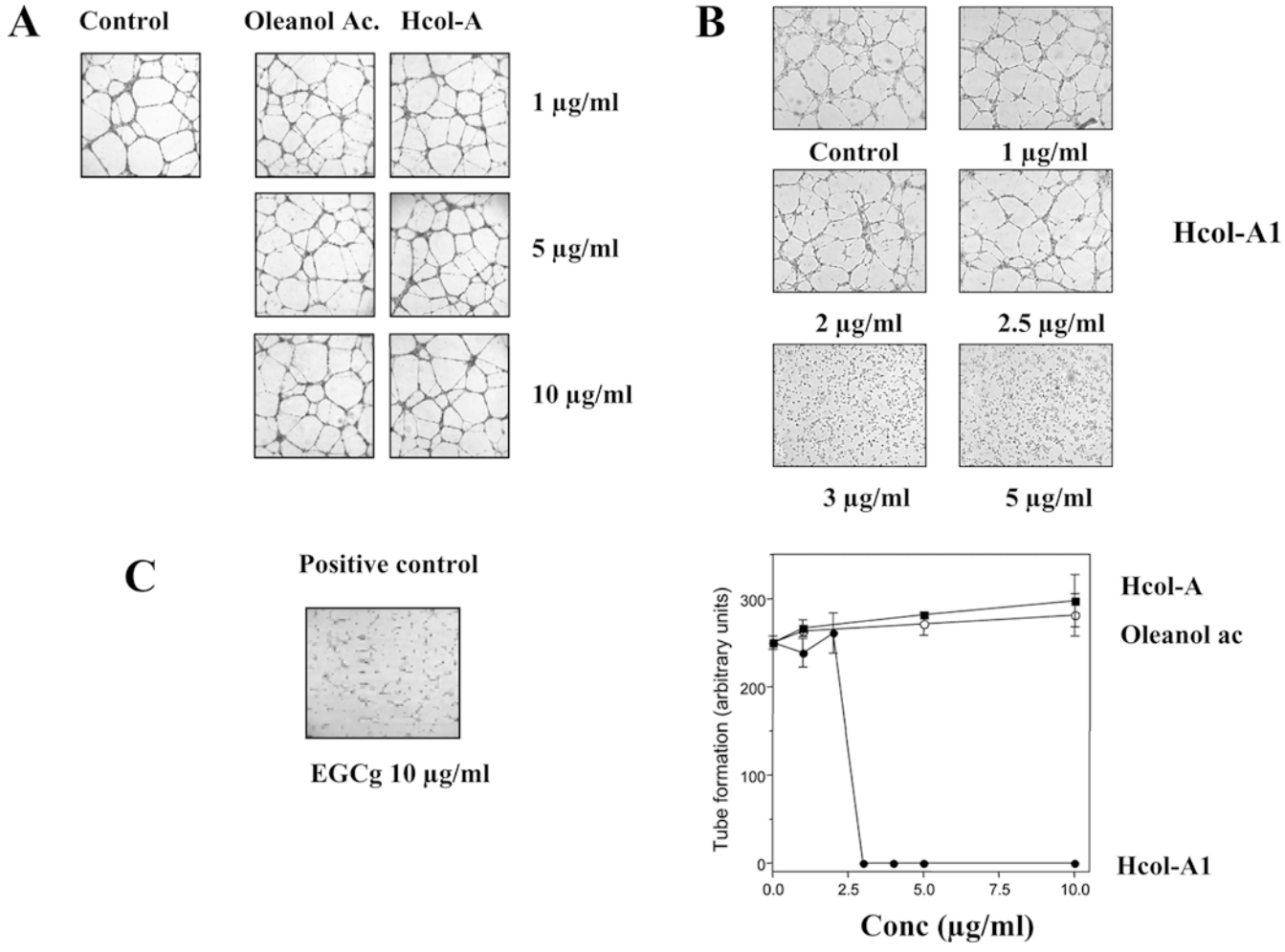


Fig. 2a–c Effects of HCol-A1 (*filled circles*), HCol-A (*filled squares*) and oleanolic acid (*open circles*) on matrigel-induced tubulogenesis. **a**, **b** Assays were conducted on HUVECs cultured for 18 h on Matrigel in the presence of specified concentrations of saponins **c**. Epigallocatechin gallate (EGCg, 10 µg/ml), the main flavonoid from green tea, was used as a positive control. The length of the capillary network was quantified as described in "Material and methods"

tain oleanane-type saponins have potential to inhibit angiogenesis

Inhibition of HUVEC tubulogenesis is associated with cholesterol sequestration

To evaluate a possible role of cholesterol in HCol-A1-mediated antiangiogenic effect, we examined whether incubation with HCol-A1 previously complexed with cholesterol, affected HUVEC tubulogenesis. When the matrigel-induced assay was conducted with HCol-A1-cholesterol complexes (5 µg/ml) instead of HCol-A (5 µg/ml), tubulogenesis inhibition was markedly decreased (Fig. 3), evidencing a critical role of cholesterol sequestration in this activity. The level of depletion in plasma membrane was evaluated by labelling with [^{3}H]cholesterol. Incubation of confluent cells with HCol-A1 for 6 h at 37°C decreased [^{3}H]cholesterol labelling by 48–53% at 5 µg/ml and 17–23% at 1 µg/ml instead of about 70% when the cholesterol-depleting agent β-cyclodextrin (2.4%) was used.

Dose-dependent and time-dependent inhibition of HUVEC viability by HCol-A1 and oleanolic acid

We have previously shown that HCol-A1 inhibits the proliferation of melanoma cells by inducing membrane-damaging effects within 6 h, non apoptotic cytoplasmic vacuolization within 24 h and inhibition of cell death within 48 h [12]. To investigate the role of cytotoxicity in inhibition of matrigel-induced tubulogenesis inhibition, HCol-A1, HCol-A or oleanolic acid were tested for their effect on cell survival after 6, 24 and 48 h continuous contact. Cell viability was measured with the WST-1 assay which measures the residual metabolic activity of treated cells. In the range of concentration used (1–10 µg/ml), HCol-A1 and, to a lesser extent, oleanolic acid exhibited significant cell toxicity. HCol-A did not significantly affect HUVECs survival (Fig. 4). HCol-A1 appeared highly toxic to HUVECs. Indeed, cell-survival was completely abolished after a 6-h continuous contact at 1 µg/ml and was decreased by 54% at 0.75 µg/ml. However, no significant effect was observed at 0.5 µg/

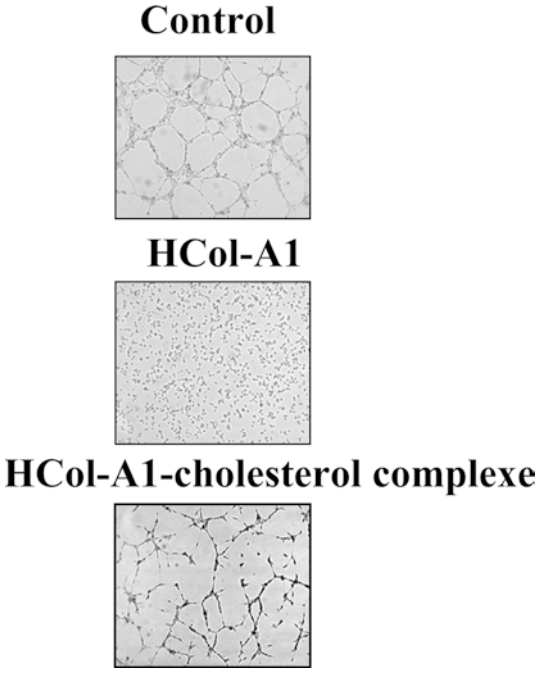


Fig. 3 Impact of cholesterol sequestration on tubulogenesis inhibition in the matrigel assay. Assay compares the effect of Hcol-A1 (5 μg/ml) and Hcol-A1-cholesterol complex (5 μg/ml) on matrigel-induced HUVEC tubulogenesis

ml. These results show a dose-dependent effect in a very close range of concentration.

The higher susceptibility of HUVECs to HCol-A1 in the present assay as compared to the former, was related to differences in testing conditions. In the latter experiment, cells were cultured in the presence of reconstituted basement (matrigel) which contains growth factors favouring cell proliferation and viability. The present assay was conducted in conditions increasing the cell-susceptibility to the drug (serum-starved medium, absence of growth factors). Oleanolic acid reduced HUVEC-survival in a dose-dependent and time-dependent manner, but a 24-h treatment at 10 μg/ml was required to abolish cell viability.

These results show that cell toxicity is involved in the inhibition of angiogenesis by HCol-A1. Structure-activity relationships indicate that Hcol-A1-mediated cell toxicity is supported by oleanolic acid and, therefore, that all oleanolic acid saponins would have anti-angiogenic potential. It is likely that, as previously demonstrated for anticancer activity [11], the number, size and type of sugars linked at C3-(OH) which define the hydrophobic/hydrophilic ratio, and therefore the cell-permeability of each saponin, would define the activity of each compound.

Activation of the Ras/MEK/ERK cascade is involved in HCol-A1-cytotoxic effects

It has previously been reported that acute depletion of membrane cholesterol increases pERK1/2 concentrations in caveola/raft and in the cytosol [25] and that cholesterol depletion activates the Ras/MEK/ERK pathway [18]. We therefore sought to determine how HCol-A1 affected pERK levels and Ras activation. Assays were conducted in confluent cells because membrane cholesterol has been reported to increase dramatically during the formation of confluent endothelial cell monolayers [21]. At 1 μg/ml, HCol-A1 induced a rapid (15 min) and significant increase pERK

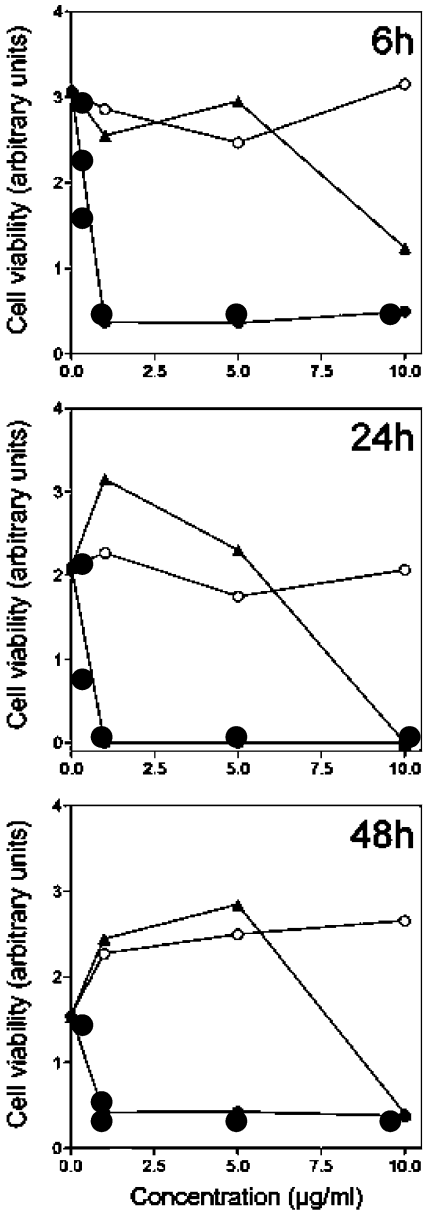


Fig. 4 Dose-dependent and time-dependent inhibition of HUVEC viability by HCol-A1 (*filled circles*), HCol-A (*open circles*) and oleanolic acid (*filled triangles*). Serum-starved HUVECs cultured without growth factors were incubated without saponin (control) or with 0.5, 0.75, 1, 5 or 10 μg/ml of HCol-A1, or 1, 5 or 10 μg/ml of HCol-A or oleanolic acid. Cell viability was assessed after 6, 24 or 48 h of continuous contact by measuring the residual metabolic activity of cells with the WST-1 assay as described in "Materials and Methods". The result is the average of three separate experiments

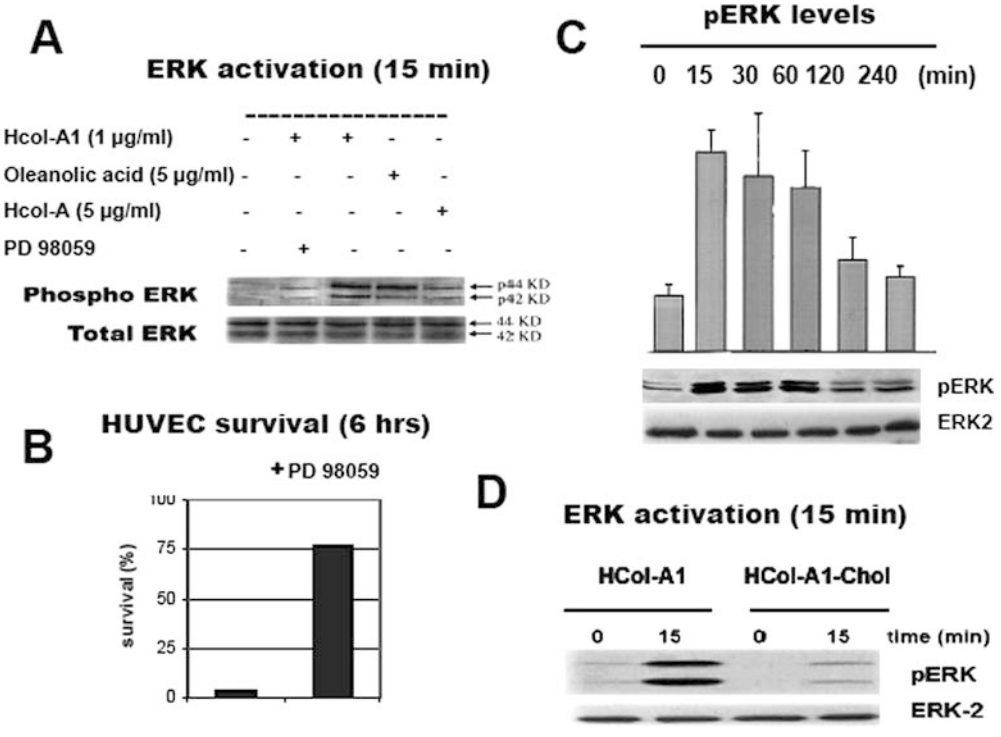
levels, whereas 5 μg/ml of HCol-A had no significant effect (Fig. 1). In contrast, at this concentration oleanolic acid strongly increased pERK levels, confirming its involvement in Hcol-A1-activity. A similar observation has been reported for ginsenoside-aglycones [26]. Cotreatment of HUVECs with PD98059, a cell-permeable and specific inhibitor of MEK1/2, the kinases immediately upstream of ERK in the MAPK cascade, abolished HCol-A1-induced ERK activation (Fig. 5a, lane 2) and increased the survival of treated cells by 77% (Fig. 5b). ERK activation by HCol-A1 was time-dependent and sustained during the 1 h following the contact with drug with a maximal effect 15 min later (Fig. 5c). In cells treated with HCol-A1-cholesterol complexes, pERK levels at 15 min were significantly decreased (Fig. 5d).

Altogether, these results indicate a direct link between ERK signalling, cytotoxicity, cholesterol sequestration and tubulogenesis inhibition by HCol-A1. Although this has to be demonstrated, it can be hypothesized that the threshold effect observed during inhibition of angiogenesis is related to the minimal level of cholesterol depletion needed to stimulate the cholesterol-sensitive feedback loop that controls ERK activation [27].

Ras is a small GTPase localized at the inner face of plasma membranes in caveolae, which regulates SAK activation [28]. Although they activate the same downstream effectors and are therefore functionally indistinguishable, the Ha-*Ras* and Ki-*Ras* isoforms mediate opposite effects in the tolerance of cells to oxidative stress. Ki-*Ras* has potent antiapoptotic activity by protecting cells against disorders associated with high intracellular levels of ROS, whereas Ha-*ras* decreases tolerance of cells to oxidative stress [29]. In HUVECs, as does β-cyclodextrin (2.4%) used as a positive control, HCol-A1, strongly activated Ha-Ras (see the doublet related to farnesylation) which appeared to be the main isoform expressed by this cell line (Fig. 6a). These findings are in agreement with those of Cuda et al. [29], who showed that in HUVECs, ERK1/2 activity is almost totally dependent of Ha-*Ras*.

We showed that cell toxicity is one of the mechanism by which HCol-A1 affects endothelial cell network formation, and that this property is directly related to acute depletion of plasma membrane cholesterol. It can be hypothesized that the over-expression of Ha-*Ras* in HUVECs contributes to increasing the susceptibility of this endothelial cell line to HCol-A1. The activation of cytochrome *c* (usually associated with oxidative stress) and the down-regulation pro-caspase-3, the inactive form of the executioner caspase-3 in Hcol-A1-treated cells (Fig. 6b), which suggests mitochondrial apoptosis

Fig. 5a–d Effect of saponins on ERK activation in subconfluent HUVECs. **a** Effect of HCol-A1 (1 μg/ml) submitted or not to cotreatment with 40 μmol of PD98059 and of HCol-A (5 μg/ml) or oleanolic acid (5 μg/ml) on ERK1/2 phosphorylation. Immunoblots were done with a mAb that recognizes the phosphorylated active form of ERK1/2 (phospho-p44/42). A pAb against total (phosphorylated and unphosphorylated) ERK1/2 was used to assess the amount of protein loaded. The positions of the phosphorylated ERK ($p42^{ERK2}$ and $p44^{ERK1}$) and total ERK1/2 (42^{ERK2} and 44^{ERK1}) are indicated by *arrows*. **b** Effect of pretreatment with PD98059 (40 μ*M*) on HUVEC survival after a 6-h continuous treatment with Hcol-A1 (1 μg/ml). Histograms represent the percentage (mean) of killed cells measured with the WST-1 reagent. **c** Time-dependent stimulation of pERK levels by HCol-A1 (5 μg/ml). The value of total ERK-2 was used as a probe of the loaded amount of protein. The intensity of activation was measured by densitometry with a TLC-2 scanner and Camag CATS-3 software (Camag, Basel, Switzerland). **d** Compared effect of HCol-A1 (5 μg/ml) or HCol-A1-cholesterol complexes (5 μg/ml) on ERK activation



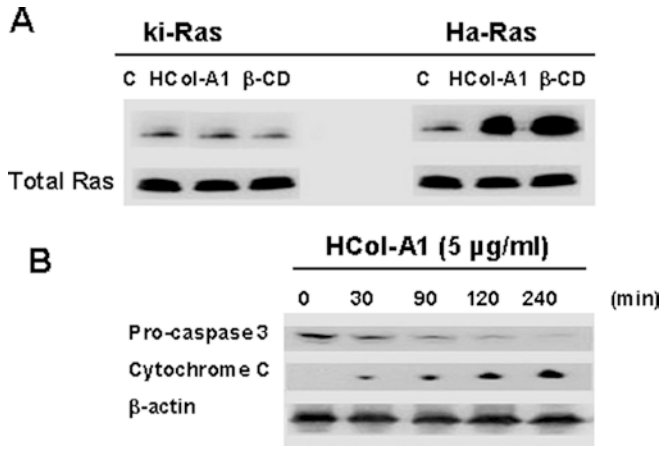


Fig. 6 Effect of saponins on Ha-Ras and Ki-Ras activation, cytochrome *c* release and pro caspase-3 activation. **a** Comparison of the effects of a 15-min incubation of subconfluent HUVECs with HCol-A1 (5 µg/ml) and the cholesterol depleting agent *β*-cyclodextrin on Ha-*Ras* and ki-*Ras* activation. Doublet corresponds to farnesyled (active) and unfarnesyled protein. *β*-cyclodextrin (2.4%) was used as a positive control. A pan-Ras Ab was used to measure the loaded amount of protein. **b** Effects of HCol-A1 (5 µg/ml) on cytochrome *c* release and procaspase-3 activation. *β*-Actin was used as a probe for the loaded amounts of protein. All results are representative of two separate experiments, each carried out in duplicate

as reported for avicins [30], support this hypothesis [18]. It is likely that caspase-9 is activated through its interaction with Apaf-1 and cytochrome *c* and that the activation of caspase-9, in turn, cleaves (and therefore activates) other caspases including the executioner caspase-3, but this remains to be demonstrated.

The ability of cancer cells to stimulate neovascularization is critical to tumour growth and metastasis. Tumours are unable to grow beyond a size of 1–2 mm and cannot form metastases, the cause of 90% of cancer deaths, without vascularization [1–3]. The present study demonstrated that HCol-A1 inhibits the formation of new vessels in the matrigel model and that Ras/MEK/ERK signalling is involved in the process. The capacity of HCol-A1 to sequester plasma membrane cholesterol appears to be the main factor affecting endothelial cell network formation. Although cell toxicity was found to play an important role in tubulogenesis inhibition in the matrigel assay, other mechanisms than cytotoxicity might be involved. Indeed, recent findings have shown that cholesterol sequestration both affects cell confluence-dependent remodelling of endothelial membranes by altering the formation of adherens junctions [21] and interacts with regulation of vascular endothelial growth factor receptor-2 (VEGFR-2) activity [31].

The stimulation of VEGFR-2 by tumour-derived VEGF represents a key event in the initiation of angiogenesis. VEGF-2 inhibitors have therefore therapeutic potential. VEGFR-2 is localized in endothelial caveolae, together with caveolin-1 recently identified as a negative regulator of VEGFR-2 activity. Endothelial cell treatment with cyclodextrin, a molecule which has been extensively shown to mediate effects similar to the effect of HCol-A1 on cholesterol membrane rafts, induces removal of VEGFR-2 and caveolin from caveolae. This dual removal increases VEGF-induced phosphorylation of VEGFR-2, but leads to the inhibition of both VEGF-induced ERK activation and endothelial cell migration. The dissociation of the VEGFR-2/caveolin-1 complex by cyclodextrin has been associated with a PP2-sensitive phosphorylation of caveolin-1 on tyrosine 14, which suggests the participation of Src family kinases in this process [30].

Wang et al. [27] have identified a cholesterol-regulated complex which combines activities of the serine/threonine phosphatase PP2A and the tyrosine phosphatase HePtP. Such a complex appears to act as (or be part of) a cholesterol-sensitive feedback loop that controls ERK activation. Src kinases are kinases that lie upstream of ERK. Although the complete chain of events triggered by HCol-A1 remains to be determined, it can be hypothesized that, in a similar manner to cyclodextrin, HCol-A1 might inhibit VEGFR-2 by modulating Src activation. Further studies are needed to verify this hypothesis which would offer new therapeutic perspectives to triterpenoid saponins, especially those promoting high cholesterol depletion.

It has recently been observed that in rats, saponins from *H. helix*, an ivy species which contains *β*-hederin, an oleanolic acid saponin whose structure is close to that of Hcol-A1, can be orally administrated without severe side effects [32]. This observation, the present findings and literature data on *β*-cyclodextrin provide a rationale to evaluate in vivo the benefits of a co-treatment associating HCol-A1 and usual chemotherapeutics (such as cisplatin or Taxol) against the aggressive proliferation of human melanoma and their pulmonary or visceral metastases.

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