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Cucurbitacin B purified from Ecballium elaterium (L.) A. Rich from Tunisia inhibits α5β1 integrin-mediated adhesion, migration, proliferation of human glioblastoma cell line and angiogenesis

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ABSTRACT

Integrins are essential protagonists in the complex multistep process of cancer progression and are thus attractive targets for the development of anticancer agents. Cucurbitacin B, a triterpenoid purified from the leaves of Tunisian *Ecballium elaterium* exhibited an anticancer effect and displayed anti-integrin activity on human glioblastoma U87 cells, without being cytotoxic at concentrations up to 500 nM. Here we show that cucurbitacin B affected the adhesion and migration of U87 cells to fibronectin in a dose-dependent manner with IC50 values of 86.2 nM and 84.6 nM, respectively. Time-lapse videomicroscopy showed that cucurbitacin B significantly reduced U87 cells motility and affected directional persistence. Cucurbitacin B also inhibited proliferation with IC50 value of 70.1 nM using Crystal Violet assay. Moreover, cucurbitacin B efficiently inhibited *in vitro* human microvascular endothelial cells (HMEC) angiogenesis with concentration up to 10 nM. Interestingly, we demonstrate for the first time that this effect was specifically mediated by α5β1 integrins. These findings reveal a novel mechanism of action for cucurbitacin B, which displays a potential interest as a specific anti-integrin drug.

1. Introduction

Cancer is the leading cause of death worldwide, accounting for 7.6 million deaths in 2008 (Ferlay et al., 2010). Glioblastoma is the most common malignant cancer of the central nervous system and remains a significant cause of death in young adults and in children. The medium survival of these patients is less than 12 months. Despite the multimodality therapy integrating surgery, radiation therapy, and chemotherapy the prognosis remains very poor (Yin et al., 2008).

Integrins are a large family of extracellular matrix (ECM) receptors, mediating the interaction of tumour cells with their microenvironment and playing an important role in glioma biology (Tabatabai et al., 2011). Integrins consist of two noncovalently bound α and β glycoprotein subunits. The combination of at least 18 α and 8 β subunits yields 24 distinct integrin dimers and determines the ligand specificity (Parsons et al., 2010; Delamarre et al., 2009). Importantly, major integrin heterodimers αvβ3 and αvβ5 (tenascin and vitronectin receptors), α5β1 (fibronectin receptor), α2β1 (collagens receptor), and α3β1, α6β4, and α6β1 (laminins receptors), along with their ECM ligands, have been found to play specific role in human glioma cancer progression and metastasis (Delamarre et al., 2009). Research of selective antagonists of integrins is nowadays a strategy for the development of more efficient therapies to completely eliminate gliomas (Tabatabai et al., 2011). For this reason biochemists and biologists have been investigating a variety of purified compound from medicinal plants (Yin et al., 2008).

Naturally occurring cucurbitacins constitute a group of tetracyclic triterpenes abundantly found in Cucurbitaceous species such as *Ecballium elaterium* (Salhab, 2013). Cucurbitacins are classified into cucurbitacin, B, D, E, I and L and their derivatives (Lee et al., 2010). Among these compounds, cucurbitacin B is the most abundant and active form (Chan et al., 2010). *Ecballium elaterium* (L.) A. Rich, a wild medicinal plant abundantly found in Tunisia, has long been used in Tunisian (Boukef, 1986), oriental and Mediterranean traditional

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medicine, for its anti-inflammatory (Greige-Gerges et al., 2007), anti-microbial (Adwan et al., 2011), and anti-cancer effects (Lavie and Szinai, 1958). The major pharmacological and biological effects of *E. elaterium* plant have been attributed to cucurbitacin B (Adwan et al., 2011). This active compound has been reported to inhibit the growth of several types of cancers, including pancreatic (Thoennissen et al., 2009), laryngeal (Liu et al., 2008), breast (Gupta and Srivastava, 2014) and lung cancers (Zhang et al., 2014). Recently, cucurbitacin B has been reported to possess antiproliferative properties on several types of tumour cells and to be active against human glioblastoma multiform (Yin et al., 2008).

In the present study, we investigate the inhibitory activities of cucurbitacin B purified from *E elaterium* leaves on several steps of cancer development. We show that this compound markedly inhibits adhesion, proliferation, migration of human glioma cancer cell lines and angiogenesis by interacting with α5β1 integrins. Results provide new evidence for the potent anti-integrin activity of cucurbitacin B on glioblastoma.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), Eagle's minimal essential medium (EMEM) and RPMI 1640 medium were purchased from GIBCO (Cergy-Pontoise, France) and fetal calf serum (FCS) from BioWhittaker (Fontenay-sous-Bois, France). Penicillin, streptomycin, human fibrinogen, human laminin and poly-L-lysine were from Sigma (St. Quentin Fallavier, France). Rat type I collagen was from Upstate (Lake Placid, NY, USA) and human fibronectin from Chemicon (Temecula, CA, USA). Human vitronectin was purified according to (Yatogho et al., 1998). Mouse monoclonal antibodies (mAbs) LM609 (anti-αvβ3) and P1F6 (αvβ5) were purchased from Chemicon. Mouse mAbs Gi9 (anti-α2β1), SAM1 (anti-α5β1) and C3VLA3 (anti-α3) and rat mAb GoH3 (anti-α6) were from Immunotech (Marseille, France). Rabbit anti-mouse and anti-rat IgG antibodies were purchased from Sigma. Matrigel™ was from BD Biosciences, Pont de Claix, France. Hexane was purchased from Fluka Chemical Co (Buchs, Swizerland).

2.2. Extract preparation and bioguided fractionation

Leaves of *Ecballium elaterium* were collected in January 2012 from a region of Sidi Thabet, area of Ariana (Northern Tunisia, latitude 36°54′45.25′N, longitude 10°06'02.10"E, altitude 30 m). Identification was made by Professor S. Ben Saad (Department of Botany, Faculty of Sciences of Tunis) according to the "Flora of Tunisia" handbook (Cuénod, 1954), and voucher specimens were deposited at the above-mentioned Laboratory of Natural Substances to serve as a future reference.

The ground dried *Ecballium elaterium* leaves were extracted with methanol using a Soxhlet extraction for 4 h. The solvent was removed via a rotary vacuum distillation at 40 °C. The total extract was dissolved in H_2O and subjected to a series of successive fractionations with hexane, dichloromethane (CH2Cl2), ethyl acetate (EtOAc), and butanol (*n*-BuOH).

The dichloromethane fraction exhibited a strong activity on U87 cell line. It was further concentrated and purified using preparative high performance liquid chromatography (HPLC). Two compounds C1 and C2 were isolated, the most active being C2. C2 was identified by LC-MS analysis by comparing of the retention times with authentic standards (Krepsky et al., 2009).

2.3. Semi-preparative liquid chromatography conditions

Separation and isolation were carried out on a semi-preparative HPLC (Agilent Technologies, Palo Alto, CA, USA). The column was a Zorbax Eclipse XDB-C8 PrepHT (H) (21.2 μm*150 mm, 5 μm) from Agilent Technologies. Sample volume of 400 μl was kept with the help of a Rheodyne 77251 injector. Detection was carried out at 230 nm with 2996 photodiode array detector. The flow rate of the mobile phase was kept at 12 ml/min. The mobile phase was composed of 0.1% acetic acid in water (A) and acetonitrile (B). The following multi-step solvent gradient was employed 0–5 min: 10% B, 5–20 min: 60% B, 20–25 min: 100% B, 25–27 min: 100% B, and 27–30 min: 10% B. Fractions were collected using a fraction collector (Agilent Technologies).

2.4. HPLC-PDA-MS analysis

LC–ESI–MS analysis was conducted in negative mode electrospray ionisation on an Agilent 1100 series HPLC systems equipped with a photodiode array detector and a triple quadrupole mass spectrometer type Micromass Autospec Ultima Pt (Kelso, UK). The separation was performed on a reverse-phase Uptisphere C18 (Interchim) (2 mm×100 mm, 5 μm particle size) column with a rate flow of 0.25 ml/min at 40 °C.

The samples (10 μl) were eluted through the column with a gradient mobile phase consisting of A (H_2O 0.1% acetic acid) and B (acetonitrile). Starting at 10% of solvent B, the proportion was programmed to reach 60% at 20 min, 100% at 25 min, and solvent B was maintained at 100% for another 2 min.

UV spectra were recorded from 190 to 800 nm and the mass spectra were recorded in negative ion mode, under the following operating conditions: capillary voltage, 3.2 kV; cone voltage, 40 V; probe temperature, 350 °C; ion source temperature, 130 °C. Mass range between 100 and 800 *m/z*. The purity of compounds was assessed using the peak purity analysis facilities of the diode array detection system of the MassLynx Software.

2.5. Cell culture

The human glioblastoma U87 cell line was routinely cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal calf serum (FCS). Human cell lines derived from colonic fibrosarcoma (HT1080) and adenocarcinoma (HT29) were cultured in DMEM containing 10% FCS. Human leukemia (K562) cells were cultured in RPMI 1640 medium containing 10% FCS. Immortalized dermal Human Microvascular Endothelial Cells (HMEC-1) (Ades et al., 1992) were obtained from the Cell Culture Laboratory in the Hôpital de la Conception (Marseille, France) and were used between passages 3 and 12. Cells were routinely grown on 0.1% gelatin-coated flasks in MCDB-131 medium containing 10% heat-inactivated FCS, 1% penicillin and streptomycin (Life Technologies, Paisley, UK), 1 mg/ml hydrocortisone (Pharmacia & Upjohn, St-Quentin-Yvelines, France) and 10 ng/ml epithelial growth factor (R & D Systems, Minneapolis, MN). Cell lines were maintained at 37 °C in a humid atmosphere 5% CO2 in air.

2.6. Cytotoxicity assay

Cytotoxicity was assessed by measuring the release of lactate dehydrogenase (LDH) activity into the culture medium upon damage of plasma membrane. Total release of LDH (100% toxicity) was obtained by adding 0.1% Triton-X100 in the incubation medium. The supernatants were collected, clarified by centrifugation 5 min at 600 g and 80 μl of supernatant were submitted to LDH-based cytotoxicity kit (Sigma) in accordance with the manufacturer's instructions.

2.7. Cell Adhesion assay

Adhesion assays were performed as previously described (Delamarre et al., 2009). Briefly, 96-well plates were coated with