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The administration of demethyl fruticulin A from *Salvia corrugata* to mammalian cells lines induces "anoikis", a special form of apoptosis

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ABSTRACT

Recently demethyl fruticulin A was identified as the major diterpenoid component of the exudates produced by the trichomes of *Salvia corrugata* leafs. Given the documented apoptotic effects of some of the other known components of the exudates from *Salvia* species, we assessed if demethyl fruticulin A, once administered to mammalian cells, was involved in the onset of apoptosis and if its biological effects were exerted through the participation of a scavenger membrane receptor, CD36. Three model cell lines were chosen, one of which lacking CD36 expression. Functional availability of the receptor, or its transcriptional rate, were blocked/reduced with a specific antibody or by the administration of vitamin E. Immunodetection of cell cytoskeletal components and tunel analysis revealed that demethyl fruticulin A triggers the onset of anoikis, a special form of apoptosis induced by cell detachment from the substrate. Impairment of CD36 availability/transcription confirmed the receptor partial involvement in the intake of the substance and in anoikis, as also sustained by FACS analysis and by the downregulation of p95, a marker of anoikis, upon blockade of CD36 transcription. However, experiments with CD36-deficient cells suggested that alternate pathways, still to be determined, may take part in the biological effects exerted by demethyl fruticulin A.

Introduction

Phytochemical studies have evidenced that several terpenoids can be obtained from plant extracts (Weckesser et al. 2007; Zheng et al. 2006), particularly from world-spread *Salvia* species (Rodriguez-Hahn et al. 1986; Rodriguez-Hahn et al. 1989). One of the major components of the triterpene fraction from *Salvia* is ursolic acid (URS) (Baricevic et al. 2001; Mathe et al. 2007; Rauter et al. 2007; Steinkamp-Fenske et al. 2007) which, along with other terpenoid-derivatives, was demonstrated to induce apoptosis in several cell types (Yim et al. 2006; Zheng et al. 2006). It is also known that, upon URS-treatment, murine macrophages release enhanced levels of interleukin 1β (IL-1 β) (Ikeda et al. 2007). This mechanism seems to be mediated by the availability of a specific membrane scavenger type B receptor, denominated FAT/CD36. This receptor takes part in the intake of LDL lipoproteins, of phosphatidyl serine and of long- chain unsaturated fatty acids, as well as in the maturation processes of dendritic cells (Puig-Kroger et al. 2006). The receptor presence was confirmed in the mitochondria (Campbell et al. 2004) and its expression, in specific cellular models, was demonstrated to decrease upon vitamin E administration (Ricciarelli et al. 2000). More recently two ixecetane diterpenoids, demethyl fruticulin A and fruticulin A (Fig. 1), previously isolated from *Salvia fruticulosa* Benth. (Rodriguez-Hahn et al. 1986; Rodriguez-Hahn et al. 1989) and *S. arizonica* Gray (Valant-Vetschera et al. 2003) were obtained with high yield along with ursolic acid/oleanoic acid from *Salvia corrugata* Vahl., an American species of the subgenus *Calosphace*, section *Corrugatae* (Bisio et al. 2008). Until now, ixecetane derivatives were demonstrated to display antimicrobial (El-Lakany et al. 1995) and cytotoxic activities (Sanchez et al. 2006; Uchiyama et al. 2003), along with a more specific trypanocidal activity (Fraga et al. 2005). Some of the properties of purified demethyl fruticulin A were investigated, revealing significant bactericidal and bacteriostatic activities against Gram-positive pathogens (Bisio et al. 2008). Although much is known on the outcomes of URS administration to mammalian tumour cells, no information is actually available about the effects exerted by

Abbreviations: URS, ursolic acid; SCO-1, demethyl fruticulin A; α-Toc, ±-α-Tocopherol, Vitamin E; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAP2, microtubule-associated protein 2; CFU, colony forming unit

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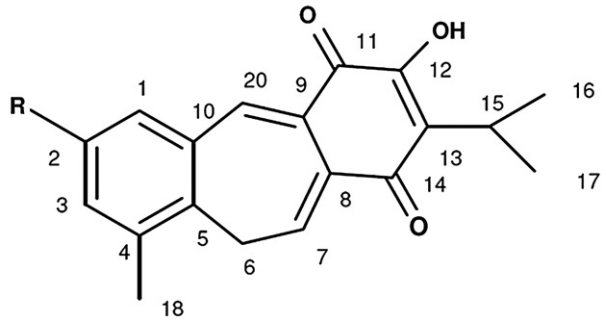

Fig. 1. Structure of Fruticulin/Demethyl fruticulin A. R (Fruticulin A) = OMe; R (Demethyl fruticulin A) = OH.

the administration of demethyl fruticulin A. Therefore we undertook this work using demethyl fruticulin A (SCO-1) purified from *Salvia corrugata* and using URS as a reference substance to which compare biological effects; moreover, in the attempt to assess a possible participation of CD36 to the effects ignited by SCO-1 exposure, we took advantage of mammalian cell cultures previously demonstrated either to exhibit or to lack CD36 expression; specifically we used transformed human epithelial cells derived from cervical carcinoma (HeLa cells) and human osteosarcoma cells (MG63 cells) which normally express CD36 (Brodeur et al. 2008; Clezardin et al. 1991), and transformed african green monkey kidney fibroblasts (COS-7 cells) which lack it (Kellner-Weibel et al. 2000; Thorne et al. 2000). Cell growth, morphology and apoptosis were evaluated, after treatment with SCO-1, to ascertain its effects and mechanisms of action in the experimental cell models used.

Materials and Methods

Salvia corrugata extract preparation and purification of demethyl fruticulin A

The extract preparation and the subsequent purification steps were performed as previously described (Bisio et al. 2008). Essentially, fresh aerial parts of *Salvia corrugata* Vahl. were obtained from the Istituto Sperimentale per la Floricultura (Sanremo, Italy) and Centro Regionale di Sperimentazione ed Assistenza Agricola (Albenga, Italy). A voucher specimen of the identified species was deposited at the Kew Herbarium (K) (Kew, Surrey, U.K). The external secreted material on the leaf surfaces was collected by rapid immersion in CH_2Cl_2 and subsequent filtration. The exudates (0.96% w/w of fresh plant) were chromographed on Sephadex LH-20 columns and eluted to fractions using a $CHCl_3$/MeOH mixture (7:3) as eluent. Specific fractions were evaporated and EtOH crystallization yielded crude demethyl fruticulin A. Quantification of demethyl fruticulin A was carried out by a reversed-phase HPLC analytical method (Bisio et al. 2008); purity grade was of 99%.

Cell cultures

Three different cell types were used: human osteosarcoma cells (MG63 cells); transformed human epithelial cells derived from cervical carcinoma (HeLa cells); and transformed african green monkey kidney fibroblasts (COS-7 cells; *Cercopithecus aethiops*; gently provided by Prof L. Zardi, Therapeutic Recombinant Proteins Unit, Advanced Biotechnology Center, Genova, Italy). Cells were maintained at 37 °C in a humidified atmosphere at 5% CO_2. All cultures were performed in monolayer using specific media supplemented with 10% fetal calf serum (FCS; GIBCO-Invitrogen, Milano, Italy). F12-medium (Biochrom AG, Berlin, Germany) was used for MG-63; Dulbecco's modified Eagle's Medium (DMEM, high glucose, EuroClone S.p.A., Milan, Italy) for COS-7 and HeLa cells. Medium was changed three times a week. Whenever needed, ursolic acid, used as a reference chemical, or demethyl fruticulin A (SCO-1) were dissolved in DMSO, added to the culture media to the tested final concentrations for the duration of the experiments (48 h) or as long as indicated. Whenever unspecified, final concentration of the purified chemicals in cell growth medium is to be intended of 50 μg/ml. Equal amounts of DMSO alone were added to all control cultures. When needed, pre-treatment of cultured cells with 100 μM α-Tocopherol (α-Toc; ±-α-Tocopherol, Sigma-Aldrich, Milano, Italy) was performed 24 h prior to the analysis or treatment. Alternatively, polyclonal anti-CD36 antibody (HPA00218, Atlas Antibodies, Sigma-Aldrich, Milano, Italy) was added to a final concentration of 2,5 μg/ml for immuno-blocking studies, one hour prior to the analysis or treatment.

Growth kinetics

Equal amounts of the different cell types cells were seeded in multiwell plates; cells were maintained in minimal media for 12 h; FCS-supplemented media were then provided to re-induce synchronous proliferation. Growth was evaluated at different time intervals using the Thiazolyl Blue staining method (MTT; Sigma-Aldrich, Milano, Italy). Absorbance readings (670-570 nm) were normalized to the values obtained from control cells at the onset of the experiment. Cell growth for the different experimental conditions was then expressed as normalized absorbance values. Alternatively, colony formation (number of colony forming units, CFU) was assessed plating equal amount of cells in standard complete media for four days and coloring colonies with methylene blue after fixation in 4% formaldehyde (PFA).

mRNA extractions, qualitative PCR and quantitative Real Time PCR reactions

Messenger RNA extraction was performed using the Perfect-Pure RNA Cultured Cell Kit (5′-Prime GmbH, Hamburg, Germany), according to the manufacturer's instructions. The SuperScript™ III First-strand synthesis system for RT-PCR (Invitrogen, Milano, Italy) was used as indicated by the manufacturer to perform standard RT-PCR reactions. Primer sets for each gene (glyceraldehyde-3 phosphate dehydrogenase, GAPDH, and CD36) were derived from published sequences (Dozin et al. 2002; Ricciarelli et al. 2000). Amplified products were resolved on 1% agarose gel, EtBr-stained and photographed under UV light. Syber green real time RT-PCR protocols were performed using the RealMasterMix SYBR ROX 2,5X (5′-Prime), with a 95 °C denaturing step for 3 minutes. Cycling conditions were set at 94 °C for 30 sec, at 60 °C for 30 sec and at 72 °C for 30 sec, for 35 cycles. Specificity of the reaction product was assessed by performing the melting curve. Human p95 primers were designed on the mature transcript (forward: 5′-CCTAGATGAGTCATTAAGGT-3′ at the exon10-exon11 junction; reverse: 5′-AGTTGGTTCCCTCTGCTCTTA-3′ at the exon11-exon12 junction) encompassing a 134 bp amplicon. Human GAPDH primers were derived from published literature (Giannoni et al. 2005). The reactions were performed on an Eppendorf Mastecycler Realplex^2 apparatus. Results were normalized to the levels of the calibrator gene GAPDH. Real time PCR runs were performed in quadruplicate for each culture conditions. For quantitative real time applications, mRNA extraction was performed 12 hours after SCO-1 treatment.