Journal of Molecular and Cellular Cardiology 69 (2014) 4–16

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Original article

Oleuropein prevents doxorubicin-induced cardiomyopathy interfering with signaling molecules and cardiomyocyte metabolism

Ioanna Andreadou [a,*], Emmanuel Mikros [a,*], Konstantinos Ioannidis [a], Fragiska Sigala [b], Katerina Naka [c], Sarantos Kostidis [a], Dimitrios Farmakis [d,e], Roxane Tenta [f], Nikolaos Kavantzas [g], Sofia-Iris Bibli [a], Evangelos Gikas [a], Leandros Skaltsounis [a], Dimitrios Th. Kremastinos [d], Efstathios K. Iliodromitis [d]

[a] Department of Pharmaceutical Chemistry, University of Athens School of Pharmacy, Athens, Greece
[b] First Department of Surgery, University of Athens Medical School, Athens, Greece
[c] Department of Cardiology, Michaelidion Cardiac Center, University of Ioannina, Ioannina, Greece
[d] Second Department of Cardiology, University of Athens Medical School, Attikon University Hospital, Athens, Greece
[e] First Department of Internal Medicine, University of Athens Medical School, Laiko Hospital, Athens, Greece
[f] Department of Nutrition and Dietetics, Harokopio University, Athens, Greece
[g] Department of Pathology, University of Athens Medical School, Athens, Greece

ARTICLE INFO

Article history:
Received 26 September 2013
Received in revised form 16 January 2014
Accepted 19 January 2014
Available online 28 January 2014

Keywords:
Oleuropein
Doxorubicin
Cardiomyopathy
Signaling molecules
Metabonomics

ABSTRACT

Oleuropein, a natural phenolic compound, prevents acute doxorubicin (DXR)-induced cardiotoxicity but there is no evidence regarding its role in chronic DXR-induced cardiomyopathy (DXR-CM). In the present study, we investigated the role of oleuropein in DXR-CM by addressing cardiac geometry and function (transthoracic echocardiography), cardiac histopathology, nitro-oxidative stress (MDA, PCs, NT), inflammatory cytokines (IL-6, Big ET-1), NO homeostasis (iNOS and eNOS expressions), kinases involved in apoptosis and metabolism (Akt, AMPK) and myocardial metabonomics. Rats were randomly divided into 6 groups: *Control*, *OLEU-1* and *OLEU-2* [oleuropein at 1000 and 2000 mg/kg in total, respectively, intraperitoneally (i.p.) for 14 days], *DXR* (18 mg/kg, i.p. divided into 6 equal doses for 2 weeks), *DXR-OLEU-1* and *DXR-OLEU-2* (both oleuropein and DXR as previously described). Impaired left ventricular contractility and inflammatory and degenerative pathology lesions were encountered only in the *DXR* group. The *DXR* group also had higher MDA, PCs, NT, IL-6 and Big ET-1 levels, higher iNOS and lower eNOS, Akt and AMPK activation compared to controls and the oleuropein-treated groups. Metabonomics depicted significant metabolite alterations in the *DXR* group suggesting perturbed energy metabolism and protein biosynthesis. The effectiveness of DXR in inhibiting cell proliferation is not compromised when oleuropein is present. We documented an imbalance between iNOS and eNOS expressions and a disturbed protein biosynthesis and metabolism in DXR-CM; these newly recognized pathways in DXR cardiotoxicity may help identifying novel therapeutic targets. Activation of AMPK and suppression of iNOS by oleuropein seem to prevent the structural, functional and histopathological cardiac effects of chronic DXR toxicity.

1. Introduction

Doxorubicin (DXR) is cardiotoxic and may lead to dose-dependent cardiomyopathy and heart failure. Chronic anthracycline-induced cardiomyopathy is associated with poor prognosis and worse survival compared to ischemic cardiomyopathy [1]. The elucidation of the mechanism of DXR-induced cardiomyopathy and its prevention is still pending [2] and the only truly effective method to prevent cardiotoxicity is a dose-limiting approach that may however compromise its chemotherapeutic properties.

Anthracycline-associated cardiotoxicity has been the subject of considerable controversy. A variety of pathways and mechanisms have been proposed including impaired expression of cardiomyocyte proteins [3], disruption of cellular and mitochondrial Ca^{2+} homeostasis [4], disruption of mitochondrial bioenergetics [5], and interference with various pro-survival kinases leading to apoptosis [6]. The most widely accepted mechanisms are the iron-mediated formation of reactive oxygen species (ROS), which promote myocardial oxidative stress [7]. However, the ROS hypothesis has been tempered by a series of negative studies, while an alternative hypothesis is still pending [8].

The natural phenolic compound oleuropein, which is present in high concentration in olives and olive tree leaves, has been shown to exert

* Corresponding authors at: Department of Pharmaceutical Chemistry, School of Pharmacy, University of Athens, Panepistimiopolis, Zografou, Athens 15771, Greece. Tel.: +30 210 7274813; fax: +30 210 7274747.
E-mail addresses: jandread@pharm.uoa.gr (I. Andreadou), mikros@pharm.uoa.gr (E. Mikros).

0022-2828/$ – see front matter © 2014 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.yjmcc.2014.01.007

potential cardioprotective actions [9], and is capable of preventing acute DXR-induced cardiotoxicity [10]. However, numerous antioxidants have been utilized for cardioprotection and, although efficient in cellular or acute animal experiments [11], they have failed to alleviate DXR cardiotoxicity in clinically relevant animal models or clinical trials [3]. The evaluation of the potential cardioprotective effects of an antioxidant against DXR-induced cardiomyopathy with a parallel investigation of several possibly implicated pathways and use of holistic — omics techniques, is generally missing. As DXR is a commonly used drug in the treatment of a wide range of cancers, such a study would potentially provide important pathogenetic and therapeutic insights. The challenge for the future is to design protocols that are cardioprotective for both the short-term and long-term effects of doxorubicin, preferably without long-term administration and without hindering the antitumor activity of the drug [12].

Bearing in mind the data/information above, we aimed to investigate the effect of oleuropein in a chronic setting of DXR-induced cardiomyopathy by addressing cardiac geometry and function, myocardial histopathology and most of the pathways that are possibly involved, including nitro-oxidative stress, inflammatory cytokines, iNOS and eNOS expressions, signaling molecules and metabonomics. Moreover we investigated if the anticancer activity of DXR is affected by oleuropein.

2. Materials and methods

2.1. Oleuropein isolation

The oleuropein used in this study was isolated from *Olea europaea* leaves as described before [9]. Briefly, air-dried and pulverized leaves were extracted with mechanical stirring for 12 h with acetone. The extract was evaporated to dryness and washed with a mixture of dichloromethane/methanol (98/2). The insoluble material was separated, dried and submitted to medium pressure liquid chromatography with Si gel 60 Merck, using dichloromethane–methanol gradient as the eluent to afford pure oleuropein. The structure elucidation of oleuropein was carried out using spectroscopic and spectrometric methods as well as comparison with literature data [13]. Specifically, 1 & 2D NMR spectra (COSY, COSYLR, HSQC-DEPT, HMBC) were recorded in deuterated methanol (CD_3OD — Merck), on a Bruker Avance III spectrometer (Bruker Biospin GmbH, Reinsteten, Germany) operating at 600.11 MHz for ^1H and at 150.11 MHz for ^{13}C, with a 5 mm inverse detection probe. The residual ^1H (3.33 ppm) and ^{13}C (49.50 ppm) signals of CD3OD were used as internal standard. 1 & 2D NMR experiments were performed with standard pulse programs, at room temperature. HRMS & HRMS/MS data were obtained by direct infusion method using a hybrid LTQ-Orbitrap Discovery Mass Spectrometer (Thermo Scientific, Bremen, Germany) equipped with an ESI probe, in positive mode. Oleuropein's purity (95%) is the maximum that can be obtained from a natural compound.

2.2. Cell line

The PC-3 cells, an androgen insensitive, p53-negative and K-Ras mutated human prostate cancer cell line, were obtained from the American Type Culture Collection (ATCC, Bethesda, MD). This cell line was grown in 75-cm^2 culture flasks using Dulbecco's modified Eagle's medium F/12 (DMEM/F-12, GIBCO/BRL) containing 5% fetal bovine serum (FBS) under standard cell culture conditions (37 °C, 5% CO_2), in a humidified atmosphere. The choice of the right culture medium is crucial, as it has been previously shown [14], that oleuropein in the commonly used media (such as MEM and DMEM), produces significant amounts of H_2O_2 which are sufficient to diminish cell viability, whereas media containing sodium pyruvate, such as DMEM/F12, abrogate the H_2O_2 produced by oleuropein.

2.2.1. MTT proliferation assay

The PC-3 cells were plated at a cell density of 500 cells/well in 96-well plates 24 h prior to treatment with the appropriate drug at various concentrations for 96 h. The anticancer actions of doxorubicin (3–100 nM) and of oleuropein (3–100 μg/ml) were assessed, alone and in co-treatment, on PC-3 cells. The cells were incubated with MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma M-5655) added directly in the medium, at a final concentration of 0.5 mg/ml, for 4 h at 37 °C. The medium was then aspirated and the cells were solubilized with the organic solvent dimethylsulfoxide (DMSO). Absorbance was determined in a VERSA max microplate reader (Molecular Devices Corporation) at 540 nm and results are presented as the percent of OD in the treated wells versus the controls [15,16].

2.3. Animals

Ninety adult male Wistar rats (300–400 g) were obtained from the Hellenic Pasteur Research Institute. All animals received proper care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals, prepared by the Academy of Sciences and published by the National Institutes of Health (Institute of Laboratory Animal Resources Commission on Life Sciences, 1996).

2.3.1. Experimental protocol

The rats were divided into 6 groups: *Control* group (n = 15), 2 ml of normal saline was injected intraperitoneally (i.p.) for 2 weeks; *OLEU-1* (n = 6) and *OLEU-2* (n = 6) groups treated with 1000 and 2000 mg/kg of oleuropein in total, respectively, given i.p. into 6 equal doses administered over a period of 2 weeks. *DXR* group (n = 21), i.p. injection of 18 mg/kg of DXR into 6 equal doses, and given over a period of 2 weeks, as previously described [17]. Oleuropein was administered at days 1, 3, 5 and so on until day 11, whereas DXR was administered at days 2, 4, 6 and so on until day 12. *DXR-OLEU-1* (n = 21) and *DXR-OLEU-2* (n = 21) groups were injected with OLEU and DXR for 2 weeks as indicated above.

Oleuropein was administered in two dosing regimens. The dose of 1000 mg/kg used in the first regimen (OLEU-1) was the same used in our previous study concerning acute-phase experiments [10]. This dose was doubled to 2000 mg/kg in the second regimen (OLEU-2), since the study period in the present study was expanded to 14 days instead of 5 used in the previous study [10] in order to investigate whether a dose–response effect existed. Oleuropein was administered i.p. since it has been shown that after a single oral dose of oleuropein 100 mg/kg is absorbed, reaching 200 ng/ml in t_{max} 2 h [18], whereas 10 min after intravenous injection of oleuropein (25 mg/kg) in rats, oleuropein was rapidly distributed with less than 25% remaining in the systemic compartment [19]. DXR is rapidly cleared from the intravascular and extracellular compartments, and accumulates in tissues in concentration considerably in excess of plasma levels. A single dose of DXR 5 mg/kg has been shown to result in a serum concentration of 0.02 + 0.01 μg/ml, after 48 h, whereas at the same time point the heart content of DXR was 7.1 + 1.5 μg/ml [20].

In order to assess the possibility of a physicochemical interaction between the two molecules, we resolved the two compounds in aqueous solution using the same ratio as for treatment and the NMR spectra were recorded and compared to the corresponding plain solutions. No shift of the NMR signals was observed for oleuropein while the DXR signals were very weak and not observable at this concentration. To further investigate the possibility of a physicochemical interaction in plasma media, DXR and OLEU were spiked in rat plasma in doses relevant to the treatment and filtered through a Nanosep Centrifugal Device (PALL Life Sciences, Ann Arbor Michigan, US) for protein retention with a molecular weight cutoff of 10 K which is able of isolating proteins from small molecules with molecular weight lower than the cutoff limit of the filter. The eluent was infused to an LTQ-Orbitrap Discovery Mass Spectrometer (Thermo Scientific, Bremen, Germany) in the positive ion