DNA Repair xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

DNA Repair

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

Hesperetin-etoposide combinations induce cytotoxicity in U2OS cells: Implications on therapeutic developments for osteosarcoma

Laura Coutinho[a], Helena Oliveira[a], Ana Rita Pacheco[a], Luis Almeida[b], Francisco Pimentel[c,d], Conceição Santos[e], José Miguel P. Ferreira de Oliveira[a,e,*]

[a] CESAM & Department of Biology, University of Aveiro, Campus Universitário de Santiago, Aveiro, Portugal
[b] Department of Pharmacology and Therapeutics, Faculty of Medicine, Porto, Portugal
[c] Department of Medical Sciences, University of Aveiro, Aveiro, Portugal
[d] Lenitudes Medical Center & Research, Santa Maria da Feira, Portugal
[e] Department of Biology, Faculty of Sciences, University of Porto, Porto, Portugal

ARTICLE INFO

Article history:
Received 3 September 2016
Accepted 21 December 2016
Available online xxx

Chemical compounds studied in this article:
Hesperetin (PubChem CID: 72281)
Etoposide (PubChem CID: 36462)
Doxorubicin (PubChem: 32874)

Keywords:
Hesperetin
Etoposide
Doxorubicin
Cell cycle
U2OS
Osteosarcoma

ABSTRACT

Osteosarcoma chemotherapy has improved survival rates, however, chemoresistance and drug toxicity still limit therapy. Drug combinations may overcome these limitations by allowing fewer chemoresistant cells to survive. The aim of this study was to evaluate the cytotoxic potential of hesperetin to osteosarcoma and to analyze the cell cycle effects of combinations of hesperetin with chemotherapeutic agents. For this, the U2OS human osteosarcoma cell line was exposed to hesperetin or hesperetin combined with etoposide or doxorubicin in defined proportions. Hesperetin was less cytotoxic compared to chemotherapeutic agents, as shown by cell growth, viability and clonogenic assays. Notwithstanding, hesperetin combined with etoposide showed additive effects on the inhibition of cell growth. Furthermore, hesperetin induced G2-phase arrest, associated with decreased gene expression of cyclins B1 and E1 and cyclin-dependent kinases 1 and 2. The combination with higher additive effect resulted in higher percentage of cells in G2-phase, showing that G2-phase arrest is associated with cytotoxicity. Moreover, hesperetin induced cytostatic effects. In conclusion, our results suggest that G2-phase arrest is an important step for hesperetin-induced cytotoxicity in U2OS cells. Hesperetin shows potential cytotoxicity when combined with etoposide, which may have implications on therapeutic developments for osteosarcoma.

1. Introduction

Osteosarcoma is the most common primary bone malignancy and is associated with radical surgery and chemotherapy [1]. The first choice of treatment is chemotherapy with agents such as doxorubicin, cisplatin, cyclophosphamide, methotrexate and etoposide, which are commonly used alone or in combination [2]. However, chemotherapy still faces major limitations, particularly the frequent occurrence of drug-resistant phenotypes. In addition, the associated cytotoxic side effects to normal tissues and organs remain a serious drawback. For instance, doxorubicin presents relevant cardiotoxicity issues, whereas etoposide is not as cardiotoxic but also not as potent as single agent against osteosarcoma [3]. Therefore, there is a pressing need to develop alternative combination treatment approaches to the current chemical treatment of osteosarcoma [4,5].

It has long been recognized that a diet rich in plant polyphenols protects against several diseases, *e.g.* cancer, cardiovascular disease, neurodegenerative diseases, diabetes and gastrointestinal disorders [6–8]. With regard to these properties, citrus flavonoids (*e.g.* hesperetin) have been studied for their activities against inflammation and cancer (*e.g.* [9]. Hesperetin, the aglycone form of hesperidin, exhibits various pharmacological activities, such as anti-inflammatory, anti-proliferative, anti-atherogenic effects and antioxidant properties [10,11]. This citrus flavanone has been shown to exert anticancer activity in many cancer cell lines such as breast cancer (*e.g.* [12], prostate cancer [13], human colon adenocarcinoma [14], and hepatocellular carcinoma [15], among others. Development of compounds or combinations that reduce the resistance of cells to drugs remains a continuing need to improve chemotherapy [16].

Abbreviations: CV, crystal violet; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

* Corresponding author at: CESAM & Department of Biology, University of Aveiro, Campus Universitário de Santiago, Aveiro, Portugal.
E-mail address: oliveira.miguel@ua.pt (J.M.P. Ferreira de Oliveira).

http://dx.doi.org/10.1016/j.dnarep.2016.12.006
1568-7864/

Noteworthy, a diversity of chemotherapeutic agents against osteosarcoma arrest the cell cycle. Specifically, the topoisomerase II poisons doxorubicin, a DNA-intercalating anthracycline, and etoposide, a non-intercalating epipodophyllotoxin, convert topoisomerase II into a DNA-damaging agent, thereby affecting the cell cycle [3,17]. In this work, the cytotoxic action of hesperetin on the U2OS osteosarcoma cell line was investigated, together with effects on the cell cycle. For this, U2OS cells were exposed to single agents (hesperetin, etoposide or doxorubicin) and combinations (hesperetin combined with etoposide or doxorubicin). The cell cycle of cells exposed to different combinations was analyzed and markers of cell cycle associated with cell cycle deregulation were identified.

2. Materials and methods

2.1. Reagents and culture media

Hesperetin, dimethyl sulfoxide (DMSO), sodium dodecyl sulfate (SDS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide and ribonuclease were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium, fetal bovine serum, antibiotics, amphotericin B and L-glutamine were purchased from Life Technologies (Carlsbad, CA, USA). Etoposide, crystal violet (CV), acridine orange were purchased from Merck Millipore (Bellerica, MA, USA). Doxorubicin was purchased from Cayman Europe (Tallinn, Estonia). Cytochalasin B was purchased from Applichem (Omaha, NE, USA).

2.2. Cell culture and exposure conditions

Human osteosarcoma cell line U2OS (American Type Culture Collection, ATCC, Vanassas, VA, USA) was a kind gift by Prof. Everardus van Zoelen. The cell line was maintained and subcultured in complete medium, *i.e.* Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 2.5 μg/ml amphotericin B. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2. Subconfluent cells were trypsinized with Trypsin-EDTA (0.25% Trypsin, 1 mM EDTA) and subcultured at a split ratio of 1:6. For exposure experiments, concentrated stock solutions of hesperetin (200 mM), doxorubicin (41.4 mM) or etoposide (60 mM) were prepared in DMSO. Final dilutions for exposure were prepared in complete medium. Final DMSO concentration was 0.1% for all conditions including control.

2.3. Cell growth and viability

Cell growth and viability was assessed by CV and MTT assays respectively. For the two assays, U2OS cells were plated at a density of 2×10^5 cells/well in 24-well plates. After overnight adhesion, cells were treated with hesperetin, etoposide, doxorubicin or combinations for 48 h. For the CV assay, cells were washed with phosphate-buffered saline (PBS) and were additionally stained with CV solution (0.2% crystal violet and 10% ethanol) for 30 min. After staining, cells were washed with distilled water and intracellular CV was dissolved in 900 μl of 1% SDS. For MTT assay, MTT reagent (final concentration 0.50 mg/ml) was added to each well at 37 °C for 4 h. After this, the medium was discarded and 900 μl DMSO were added. For the CV and MTT assays, 24-well plates were shaken for 1 h and absorbance was measured at 570 nm using a Synergy HT Multi-mode Microplate Reader (BioTek Instruments, Winooski, VT, USA). For cells exposed to combinations of hesperetin and chemotherapeutic agents isobologram analysis was performed.

2.4. Clonogenic assay

U2OS cells (50 cells/well) were seeded in 6-well plates and allowed to adhere overnight at 37 °C. Cells were then treated with hesperetin, etoposide, doxorubicin and combinations as indicated and incubated for 48 h exposure time. After exposure, cells were washed with PBS, detached by trypsinization, suspended in complete medium and transferred to 6-well plates (50 cells/well). After 2-weeks incubation the cells were fixed and stained as for CV assay with CV solution for 30 min. The plate was washed with distilled water and allowed to dry overnight. Colony image was acquired in a G:BOX CHEMI HR-16 BioImaging system (Syngene, Frederick, MD, USA) and clonogenic assay parameters colony number and area were quantified with ImageJ.

2.5. Cell cycle analysis

Cell cycle analysis was performed as previously [18]. Briefly, 6×10^5 U2OS cells were seeded per well in a 6-well plate and allowed to adhere overnight at 37 °C. After adhesion, cells were treated with hesperetin, etoposide, doxorubicin and combinations as indicated and incubated for an additional 48 h for exposure. Following trypsinization, cells were centrifuged at 600*g* for 10 min, and the pellet was washed in PBS. The cells were recentrifuged as previously (600*g*, 10 min) and the cell pellets were resuspended in 1 ml of 85% ethanol at 4 °C after which they were stored at −20 °C until analysis. For cell cycle analysis, samples were centrifuged at 600*g* for 10 min, and the cell pellets were resuspended in 0.8 ml of PBS. Finally, cell suspensions were filtered through a 35-μm nylon mesh, and ribonuclease (final 50 μg/ml) and propidium iodide (final 50 μg/ml) were added. A Coulter Epics XL Flow Cytometer (Beckman Coulter, Hialeah, FL, USA) equipped with an argon laser (15 mW, 488 nm) was used to measure relative fluorescence intensity of PI-stained nuclei. Acquisitions were performed using SYSTEM II software v. 3.0 (Beckman Coulter, Hialeah, FL, USA). For each sample, the number of events analyzed was approximately 5000. Cell cycle analysis was then conducted based on the histogram outputs, and frequency results were obtained with the software FlowJo (Tree Star, Ashland, OR, USA).

2.6. RNA extraction and qPCR

Gene-specific primers (Table 1) were designed using Primer3 design [19] and tested for specificity using UCSC In-Silico PCR tool (http://genome.ucsc.edu/cgi-bin/hgPcr?command=start). RNA was extracted using the TRIzol method. Organic phase separation was achieved in Phase Lock Gel Heavy tubes (5 PRIME Inc., Boulder, CO, USA). The aqueous phase was mixed with 1 vol. 70% ethanol and RNA was purified using NZY Total RNA Isolation kit (NZYTech, Lisbon, Portugal), including DNase I incubation. For cDNA synthesis, 1 μg total RNA was reverse-transcribed with 2.5 μM Oligo (dT)18, using the NZY First-Strand cDNA Synthesis Kit (NZYTech,

Table 1
Oligonucleotide primer sequences used for qPCR.

Gene	Oligonucleotide primer sequence (5′-3′)
CCNB1 (cyclin B1)	F: GCTGAAAATAAGGCGAAGATCAA
	R: ACCAATGTCCCCAAGAGCTG
CCNE1 (cyclin E1)	F: CAGCCTTGGGACAATAATGC
	R: GAGGCTTGCACGTTGAGTTT
CDK1	F: GGGTAGACACAAAACTACAGGTCAA
	R: GGAATCCTGCATAAGCACATC
CDK2	F: ACCTCCCCTGGATGAAGATG
	R: AGATGGGGTACTGGCTTGGT
GADPH	F: ACACCCACTCCTCCACCTTT
	R: TACTCCTTGGAGGCCATGTG

Please cite this article in press as: L. Coutinho, et al., Hesperetin-etoposide combinations induce cytotoxicity in U2OS cells: Implications on therapeutic developments for osteosarcoma, DNA Repair (2016), http://dx.doi.org/10.1016/j.dnarep.2016.12.006