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Evaluation of the antioxidant properties of carexanes in AGS cells transfected with the *Helicobacter pylori*'s protein HspB

Elisabetta Buommino [a, *, 1], Brigida D'Abrosca [b, 1], Giovanna Donnarumma [c], Annamaria Parisi [c], Monica Scognamiglio [b], Antonio Fiorentino [b, 1], Antonio De Luca [d, 1]

[a] Department of Pharmacy, University of Naples Federico II, 80131, Italy
[b] Department of Environmental Biological and Pharmaceutical Sciences and Technologies, Università degli Studi della Campania Luigi Vanvitelli, 81100 Caserta, Italy
[c] Department of Experimental Medicine, Section of Microbiology and Clinical Microbiology, Università degli Studi della Campania Luigi Vanvitelli, Naples, 80138, Italy
[d] Department of Mental and Physical Health and Preventive Medicine, Section of Human Anatomy, Università degli Studi della Campania Luigi Vanvitelli, Naples, 80138, Italy

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ABSTRACT

Naturally derived compounds represent a potential source of pharmacologically active drugs able to contrast different diseases, including gastric cancer, a multifactorial disease, in which the important role played by *H. pylori* infection has been demonstrated. Carexanes, stilbene derivatives, isolated from plants of the *Carex distachya* Desf., are unusual secondary metabolites with a tetracyclic skeleton arising from a cyclization of prenylstilbenoid precursors.

In this study we firstly showed the ability of three purified carexanes CxB, CxG, and CxI to enhance the antioxidant response of AGS cells and to contrast the effect of the *H. pylori's* protein HspB. Among them CxI was the molecule that best modified the expression of genes involved in the antioxidant response. In particular, CxI was able to reduce Keap-1 gene expression and induce NQO1 gene expression, both at 4 and 24 h in AGS cells, as showed by real time PCR. Nrf2 induction was evident only at 24 h. Interestingly, the effect of CxI was stronger in HspB-transfected AGS cells, where Keap-1 gene expression was nearly abrogated. Finally, we demonstrated that CxI was able to reduce also COX-2 gene expression in HspB-transfected AGS cells, compared with untreated HspB-transfected cells, both at 4 and 24 h.

This study first report that carexanes might represent candidate molecules able to contrast the deleterious effect of HspB protein but also to reduce the inflammatory process induced by *H. pylori* infection.

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1. Introduction

Gastric cancer is one of the most common malignancies in the world, although the incidence and mortality rate have been decreasing in recent decades. The association between *H. pylori* and gastric cancer has attracted great interest worldwide. The International Agency for Research on Cancer (IARC) identified *H. pylori* as a "group 1 (definite carcinogen)" in 1994 on the basis of the results of epidemiologic studies [1]. Gastric mucosal infection with *H. pylori* is accompanied by infiltration of neutrophils, and activated inflammatory cells are known to produce oxygen radicals. Davies et al. have reported an increase of oxygen radical production in both the duodenal and gastric pyloric mucosa after infection with *H. pylori* [2].

Reactive oxygen species (ROS) activate signaling pathways involved in cell proliferation and migration, as well as damage macromolecules like DNA and proteins, leading to mutations [3]. Antioxidants counteract oxidative stress, reducing ROS levels in the intracellular environment; thus the beneficial impact of antioxidant molecules in reducing cancer risk is well appreciated, since they may confer a survival advantage [4,5]. Nrf2 (nuclear factor-erythroid 2-related factor 2) is an important regulator of the

* Corresponding author. Department of Pharmacy, University of Naples Federico II, Via Domenico Montesano, 49, 80131, Italy.
E-mail address: elisabetta.buommino@unina.it (E. Buommino).
[1] The first two authors, E.B. and D.B., and the last two (A.F and A.DL) equally contributed to this work.

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cellular response against oxidative stress [6]. It coordinates induction of genes encoding numerous antioxidants, phase II detoxifying enzymes and related proteins such as catalase, superoxide dismutase (SOD), NAD(P)H:quinone oxidoreductase-1, heme oxygenase-1 (HO-1), glutathione S-transferase (GST), glutathione peroxidase, and others. When oxidative stress occurs, Nrf2 is released from its repressor molecule, Keap1 (Kelch-like ECH-associated protein 1), and translocates to the nucleus to promote the transcription of the antioxidant and detoxifying enzyme [7].

During the past decades, a widespread search has been launched to identify new anti-cancer therapies from natural sources. Herbs, medicinal plants, spices, vegetables have showed to represent a potential source to combat various diseases, including gastric cancer [8–10].

It is now accepted that *Helicobacter pylori* significantly increases the risk of developing atrophic gastritis, peptic-ulcer disease and gastric carcinoma [1,11]. We recently reported evidences that the HspB protein of *H. pylori* interferes in the Nrf2/Keap-1 pathway [12].

The increasing therapeutic failures against *H. pylori* infection has determined the need to develop new drugs. 3,5,4-trihydroxy-*trans*-stilbene known as *trans*-resveratrol, a phenolic compound present in grapes, wine, peanuts, and other food products, has antioxidant properties, exerts a potent anti carcinogenic activity and protective effects against atherogenesis and cardiovascular diseases [13]. Moreover it has been reported to possess anti-*H. pylori* activity *in vitro* [14]. Recently, trans-resveratrol has also been tested on *H. pylori* CagA + strains from patients with gastric carcinoma, displaying a strong antibacterial activity [15].

Plants of the *Carex* genus produce oligostilbenes comprising from two to four monomers of resveratrol, most of them showing antimicrobial activity [16,17]. Carexanes are secondary metabolites isolated only from *Carex distachya* Desf., so far characterized by an unusual tetracyclic skeleton arising from a cyclization of prenylstilbenoid precursors [18]. Few articles report the isolation of prenylated stilbenes from natural sources. These compounds have been identified as cytotoxic against ovarian cancer cell lines [19].

Here we demonstrated that carexanes enhance the antioxidant response of HspB-transfected human gastric epithelial (AGS) cells reducing the risk of gastric cell transformation.

2. Materials and methods

2.1. General experimental procedures

Optical rotations were measured on a Perkin–Elmer 141 in MeOH solution. UV spectra were performed on UV-1700 Shimadzu spectrophotometer in MeOH. NMR spectra were recorded at 300.03 MHz for ^{1}H and 75.45 MHz for ^{13}C on a Varian Mercury300 spectrometer Fourier transform NMR in CD_3OD or $CDCl_3$ solutions at 25 °C. Analytical TLC was performed on Merck Kiesel gel 60 F254 plates with 0.2 mm layer thickness. Spots were visualized by UV light or by spraying with H_2SO_4/AcOH/H_2O (1:20:4). The plates were then heated for 5 min at 110 °C. Preparative TLC was performed on Merck Kiesel gel 60 F254 plates, with 0.5 or 1.0 mm film thickness. Column chromatography (CC) was performed on Merck Kieselgel 60 (70–240 μm), Merck Kieselgel 60 (40–63 μm), Sephadex LH-20®.

2.2. Plant material

Plants of *Carex distachya* Desf. (Cyperaceae) were collected in June 2012, in the vegetative state, in the Nature Reserve of Castelvolturno, near Caserta (Italy), and identified by Dr Assunta Esposito of Università degli Studi della Campania Luigi Vanvitelli. A voucher specimen (CE278) has been deposited at the Herbarium of the Dept. of Environmental, Biological and Pharmaceutical Sciences and Technologies of Università degli Studi della Campania Luigi Vanvitelli.

2.3. Extraction and isolation of the metabolites

Fresh roots of *Carex distachya* (3.0 kg) were extracted with hexane for 5 days at 4 °C in the dark. The organic solutions were distilled under reduced pressure by a Rotavapor® to obtain a crude extract (54.0 g), which was chromatographed on CC SiO_2 eluting with hexane/EtOAc increasing polarity solutions and collecting three fractions (A–C).

Fraction A, eluted with hexane-EtOAc(9:1) was chromatographed on Sephadex LH-20® eluting with hexane-$CHCl_3$-MeOH (3:1:1) to obtain a fraction which was purified by SiO_2*flash*-CC eluting with hexane-EtOAc (7:3) to obtain pure carexane I (CxI, 25.0 mg).

Fraction B, eluted with hexane-EtOAc(9:1) re-chromatographed on Sephadex LH-20® eluting with hexane-$CHCl_3$-MeOH (3:1:1) to obtain a fraction which was purified by SiO_2TLC eluting with hexane-EtOAc (4:1) to obtain pure carexaneB (CxB, 10.2 mg).

Fraction C, eluted with hexane-EtOAc(9:1) and re-chromatographed on Sephadex LH-20® eluting with hexane-$CHCl_3$-MeOH (1:1:1) gave pure carexane G (CxG, 23.6 mg).

2.4. Cell cultures, stable DNA transfection and treatments

AGS human gastric epithelial cells (American Type Culture Collection, Manassas, VA, USA) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 50 mg/mL penicillin–streptomycin in an atmosphere of 5% CO_2 at 37 °C.

Stable transfected AGS cells were generated with mammalian expression vector for pcDNA3T7Tag-HspB (Invitrogen - Carlsbad, California USA) and pcDNA3 empty vector as control using Lipofectamine (Invitrogen) according to the manufacturer's instruction as previously described [12,20]. Afterwards, the cells were plated in 60 mm dishes (1×10^6 cells/dish) 24 h before transfection. Stably transfected cells were selected with 800 μg/ml G418. Experiments were performed on AGS cells stably transfected with HspB and cultured with complete DMEM supplemented with 800 μg/mL G418 for at least 1 month. Semi confluent AGS and stable transfected AGS cells were treated with different concentrations of CxB, CxG, CxI (50-75-100 μg/mL) for 4 and 24 h, to better analyze the effect of carexanes on gene expression after a short and longer treatment. Any differences was observed between control cell and pcDNA3 empty vector, in terms of gene expression modification, as also previously demonstrated [12]. Thus, in this study control cells refers to untreated AGS cells.

2.5. Morphological analysis

The morphological features of AGS cells treated with CxB, CxG and CxI were defined by phase-contrast microscopy (Olympus CDK40) at 20× magnification.

2.6. MTT cell proliferation assay

AGS cells (4×10^3) treated or not with CxB, CxG, CxI(50-75-100 μg/mL) were grown in microplates (tissue culture grade, 96 wells, flat bottom) in a final volume of 100 μl DMEM, at 37 °C and 5% CO_2. After 24 h, 10 μl of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) labelling reagent (Roche Diagnostics, Basel, Switzerland; final concentration 0.5 mg/ml) were added to each well. After 4 h, 100 μl of the solubilisation solution

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