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Original article

The apoptotic and autophagic properties of two natural occurring prodrugs, hyperoside and hypoxoside, against pancreatic cancer cell lines

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ABSTRACT

Pancreatic cancer is only the 12th most common cancer, but the fourth leading cause of cancer-related deaths in the world. This is due to late prognosis, poor response to chemotherapy and early metastases. Natural prodrugs may play an important role in the treatment of pancreatic cancer. The main aim of this study was to determine the cytotoxicity of five natural prodrugs, namely harpagoside, hyperoside, hypoxoside, oleuropein and polydatin, by investigating apoptosis and autophagy as possible mechanism/s of action. Hypoxoside and hyperoside have shown selective cytotoxicity at IC50 values of ~25 and 50 μM against INS-1 and MIA PaCa-2 pancreatic cancer cells, respectively. Hypoxoside and hyperoside induced G2/M phase arrest and caspase-3 activation in INS-1 and MIA PaCa-2 cells, respectively. Hoechst/phalloidin staining confirmed morphological changes, including condensed chromatin multinucleation, membrane blebbing and loss of cytoskeletal arrangement in INS-1 and MIA PaCa-2 cells. Acridine orange staining was absent in INS-1 (hypoxoside) and MIA PaCa-2 (hyperoside) treated cells, whereas LC3B expression was not significantly increased. INS-1 and MIA PaCa-2 treated cells favour the cell death pathway, apoptosis, over the cell survival pathway, autophagy.

1. Introduction

The pancreas is an important organ with both endocrine and exocrine functions. The exocrine function involves the secretion of digestive enzymes into the small intestine, whereas the endocrine function involves the secretion of hormones, including insulin, glucagon and somatostatin [1]. About 95% of pancreatic cancer targets the exocrine function, and five percent the endocrine function. The former is malignant and fast growing, whereas the latter is benign and slow growing [1,2]. Pancreatic ductal adenocarcinoma accounts for more than 90% of pancreatic cancer. Although it is only the 12th most common cancer, it is the fourth leading cause of cancer-related deaths in the world due to low survival rates [3]. It evolves from pancreatic intraepithelial-, mucinous cystic- and intraductal papillary neoplasias, which are microscopic premalignant pancreatic lesions associated with pancreatic ducts [4,5].

Pancreatic cancer is associated with poor prognosis due to rapid progress with few symptoms, advanced stage at time of diagnosis, poor response to chemotherapy, and early metastases [3,4,6]. The one- and five-year survival rates for all stages of pancreatic cancer are approximately 18% and 4%, respectively [3]. The median survival from diagnosis without treatment is between three and six months, and median survival from diagnosis with resectional therapy and adjuvant therapy is about 23 months [4]. It is predicted that there will be a 55% increase in new pancreatic cancer cases by 2030 [7].

The major challenge of any antitumour drug is the specific targeting and destruction of malignant cells with minimal or no effect on normal/healthy cells. Enzymes are naturally overexpressed in the tumour microenvironment and can selectively activate enzyme-responsive prodrugs [8]. It is estimated that

Abbreviations: AO, acridine orange; AP-1, activating protein-1; COX-2, cyclooxygenase-2; CREB, cAMP response element binding protein; DMSO, dimethyl sulfoxide; DPBS, Dulbecco's phosphate buffered saline; EDTA, ethylene diaminetetraacetic acid; ERK, extracellular signal-regulated kinases; FITC, fluorescein isothiocyanate; LC3B, light chain 3B; mTOR, mammalian target of rapamycin; MTT, 3-(4,5-dimethyl-2-thiazolul)-2,5-diphenyl-2H-tetrazolium bromide; NF-κB, nuclear factor-kappa B; PARP, poly (ADP-ribose) polymerase; PI3K, phosphatidyl-3-kinase; STAT3, signal transducer and activator of transcription 3.

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between 5 and 7% of approved drugs worldwide can be classified as prodrugs. In 2001/2002, about 15% of new approved drugs were prodrugs [9]. Advantages of prodrugs over parent drugs include increased solubility, bioavailability, stability and selectivity, therapeutic specificity, and fewer side effects [10,11]. Prodrugs can be activated via enzymatic-mediated hydrolytic/oxidoreductive- or chemical nonenzymatic processes. Examples of hydrolytic enzymes used in prodrug activation include β-glucuronidase, β-galactosidase, β-lactamase, alkaline phosphatase and matrix metalloproteinases, to name a few [10].

The main aim of this study was to investigate the cytotoxic properties of five naturally occurring prodrugs, namely harpagoside, hyperoside, hypoxoside, oleuropein and polydatin, against INS-1 and MIA PaCa-2 pancreatic cancer cells. Furthermore, the apoptotic and autophagic mechanism/s of action were elucidated.

2. Materials and methods

2.1. Chemicals and reagents

Cleaved caspase-3 (Asp175) and light chain 3B (LC3B; D11) XP (R) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Coulter® DNA Prep™ reagents kit, goat anti-rabbit IgG (H + L chain specific) and rabbit IgG isotype, both labeled with fluorescein isothiocyanate (FITC) conjugate, and IsoFlow™ EPICS™ sheath fluid were purchased from Beckman Coulter (CA, USA). IntraPrep™ permeabilizing reagent was purchased from Immunotech (Marseille, France). Harpagoside, hyperoside, oleuropein, polydatin, 3-(4,5-dimethyl-2-thiazolul)-2,5-diphenyl-2H-tetrazolium bromide (MTT), β-glucosidase (from almonds) β-galactosidase (from *Aspergillus oryzae*), chloroquine diphosphate salt, melphalan, bisBenzimide H33342 trihydrochloride, phalloidin tetramethylrhodamine B isothiocyanate and quercetin were purchased from Sigma (St. Louis, MO, USA). Acridine orange (AO) was purchased from BDH Chemicals Ltd. (Poole, England). Hypoxoside and rooperol, purified using the method of Kruger et al. [12,13], were kindly donated by Dr. Carl F. Albrecht.

2.2. Cell culture conditions

Rat pancreatic insulinoma (INS-1) was kindly donated by Prof Guy Ritter (University of Bristol, England), and human pancreatic ductal adenocarcinoma (MIA PaCa-2) was purchased from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan), respectively. INS-1 cells were routinely maintained in RPMI1640 medium (HighClone Laboratories, Inc., South Logan, Utah, USA) supplemented with 10% fetal bovine serum (HighClone Laboratories, Inc., South Logan, Utah, USA) in the presence of penicillin-streptomycin (100 U/mL, Lonza, BioWhittaker, Verviers, Belgium). MIA PaCa-2 cells were routinely maintained in Eagle's minimum essential medium (HighClone Laboratories, Inc., South Logan, Utah, USA) supplemented with 10% fetal bovine serum and 1% non-essential amino acids (Sigma, St. Louis, MO, USA) in the presence of penicillin-streptomycin. Cells were incubated in a humidified 5% CO_2 incubator at 37 °C.

2.3. Cytotoxicity and IC50 determination

INS-1 and MIA PaCa-2 cells were seeded in 96-well plates at cell densities of 10,000 and 6000 cells/100 μL/well, respectively. Cells were allowed to attach overnight at 37 °C and 5% CO_2 in a humidified incubator. Stock concentrations of hyperoside, harpagoside, hypoxoside, oleuropein and polydatin were prepared in dimethyl sulfoxide (DMSO) at 50 mM. Working concentrations of 200 μM were prepared in complete medium and dilutions, ranging between 12.5 and 100 μM, tested. β-Galactosidase (hyperoside) and β-glucosidase (harpagoside, hypoxoside, oleuropein and polydatin) at 100 μg/mL, were added to the prodrugs to cleave the *O*-glycosidic bonds of galactose and glucose subunits, respectively. Each 96-well plate was divided into top and bottom halves. The top half was treated with the prodrug in the absence of enzyme, whereas the bottom half was treated with the prodrug in the presence of enzyme. Melphalan was used as positive control at working concentrations ranging between 1.56 and 200 μM. Cells were treated for 48 h at 37 °C and 5% CO_2 in a humidified incubator.

Cell viability was determined using the MTT assay as described by Holst-Hansen and Brünner [14], and the absorbance read at 540 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA).

2.4. DNA cell cycle arrest for apoptosis

INS-1 and MIA PaCa-2 cells were seeded in 12-well plates at cell densities of 250,000 and 150,000 cells/mL/well, respectively. Cells were left to attach overnight at 37 °C and 5% CO_2 in a humidified incubator. INS-1 cells were treated with 12.5, 25 and 37.5 μM of hypoxoside, and MIA PaCa-2 cells were treated with 25, 50 and 75 μM of hyperoside. β-Galactosidase and β-glucosidase (final concentration: 100 μg/mL) were added to the hyperoside and hypoxoside treated wells, respectively. For a positive control, INS-1 and MIA PaCa-2 cells were treated with 30 and 40 μM melphalan, respectively. Cells were treated for 48 h at 37 °C and 5% CO_2 in a humidified incubator. After 48 h, the spent media were transferred to polypropylene tubes, cells washed with Dulbecco's Phosphate Buffered Saline (DPBS without Ca^{2+}/Mg^{2+}, 1 mL) and trypsinized with 200 μL trypsin-ethylenediaminetetraacetic acid (EDTA; Biowest, Nuaille, France) for 10–15 min at 37 °C. Cells were resuspended in 1 mL DPBS and transferred to the corresponding tubes. Tubes were centrifuged at 500 × *g* for 5 min at room temperature. Supernatants were decanted and the cells washed with DPBS (1 mL). The Coulter® DNA Prep™ reagents kit was used to determine DNA cell cycle arrest as described in the kit's protocol with a few modifications. In brief, cells were lysed using the lysis reagent (50 μL; containing 0.1% NaN_3, non-ionic detergents, saline and stabilizers), vortexed and incubated for 5 min at room temperature. Propidium iodide (300 μL; 50 μg/mL) was added to each tube and the cells incubated in the dark for 15 min at 37 °C. Cells were immediately analyzed on a Beckman Coulter Cytomics FC500 flow cytometer (FL, USA) with a 488 nm argon laser. Red fluorescence was measured in the FL3 channel and a minimum of 10,000 events per sample recorded.

2.5. Caspase-3 activation for apoptosis

INS-1 and MIA PaCa-2 cells were seeded as described in Section 2.4. INS-1 cells were treated with 25 and 30 μM hypoxoside and melphalan, respectively. MIA PaCa-2 cells were treated with 50 and 40 μM hyperoside and melphalan, respectively. β-Galactosidase and β-glucosidase (final concentration: 100 μg/mL) were added to the hyperoside and hypoxoside treated wells, respectively. Cells were treated for 48 h at 37 °C and 5% CO_2 in a humidified incubator. After 48 h, the spent media were transferred to polypropylene tubes, cells washed with DPBS (without Ca^{2+}/Mg^{2+}, 1 mL) and trypsinized with 200 μL trypsin-EDTA for 10–15 min at 37 °C. Cells were resuspended in 1 mL DPBS and transferred to the corresponding tubes. Tubes were centrifuged at 500 × *g* for 5 min at room temperature. Supernatants were decanted and the cells washed with DPBS (1 mL), as described above, to remove any traces of trypsin. Cells were fixed and permeabilized using the IntraPrep™ permeabilizing reagent as described in the kit's protocol. After permeabilization, cells were washed using cold incubation buffer (0.5% BSA in DPBS), supernatant discarded, and cells blocked