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Calix[6]arene bypasses human pancreatic cancer aggressiveness: Downregulation of receptor tyrosine kinases and induction of cell death by reticulum stress and autophagy

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ABSTRACT

Pancreatic cancer ranks fourth among cancer-related causes of death in North America. Minimal progress has been made in the diagnosis and treatment of patients with late-stage tumors. Moreover, pancreatic cancer aggressiveness is closely related to high levels of pro-survival mediators, which can ultimately lead to rapid disease progression, resistance and metastasis. The main goal of this study was to define the mechanisms by which calix[6]arene (CLX6), but not other calixarenes, efficiently decreases the aggressiveness of a drug resistant human pancreas carcinoma cell line (Panc-1). CLX6 was more potent in reducing Panc-1 cell viability than gemcitabine and 5-fluorouracil. In relation to the underlying mechanisms of cytotoxic effects, CLX6 led to cell cycle arrest in the G0/G1 phase through downregulation of PIM1, CDK2, CDK4 and retinoblastoma proteins. Importantly, CLX6 abolished signal transduction of Mer and AXL tyrosine kinase receptors, both of which are usually overexpressed in pancreatic cancer. Accordingly, inhibition of PI3K and mTOR was also observed, and these proteins are positively modulated by Mer and AXL. Despite decreasing the phosphorylation of AKT at Thr308, CLX6 caused an increase in phosphorylation at Ser473. These findings in conjunction with increased BiP and IRE1-α provide a molecular basis explaining the capacity of CLX6 to trigger endoplasmic reticulum stress and autophagic cell death. Our findings highlight CLX6 as a potential candidate for overcoming pancreatic cancer aggressiveness. Importantly, we provide evidence that CLX6 affects a broad array of key targets that are usually dysfunctional in pancreatic cancer, a highly desirable characteristic for chemotherapeutics.

1. Introduction

Pancreatic cancer is the fourth leading cause of cancer-related deaths in North America [1]. This form of cancer is extremely aggressive; it has a high capacity for local invasion and distant metastases even during early disease stages [2]. In addition, the high heterogeneity of pancreatic cancer, caused by the deregulation of multiple signaling pathways, culminates in the inherent or acquired resistance to chemotherapy, radiotherapy, or both [3,4]. Due to this, current therapeutic approaches for treating pancreatic cancer are rather inefficient and unable to revert the aggressive nature of this disease. Unsurprisingly, the lack of new chemotherapeutic strategies is reflected in the small increase in pancreatic cancer survival over the past 30 years [5]. From this perspective, the search for new drugs for pancreatic cancer therapy and the definition of molecular mechanisms underlying their actions are crucial for improving the efficacy of pancreatic cancer treatment. Calix[*n*]arenes have emerged as promising anti-tumoral agents.

Calix[*n*]arenes are macrocyclic compounds of phenolic units linked by methylene or sulfur groups at the 2,6-positions [6]. These compounds have been widely used for their diverse biological properties. Calixarenes have shown potential as enzymatic inhibitors [7–9], anticoagulants and antithrombotics [10], antivirals [11], antimicrobials [12–14] and anticancer drugs [15–19]. Moreover, calixarenes can be synthesized in large amounts, and can easily be

Abbreviations: CLX6, Calix[6]arene; CDKs, cyclin-dependent kinases; RB, retinoblastoma; PIM, proviral integration site for the moloney murine leukemia virus; AXL, tyrosine-protein kinase receptor UFO; Mer, tyrosine-protein kinase receptor; PI3K, phosphoinositide 3-kinase; AKT, a serine/threonine-specific protein kinase, also known as Protein Kinase B (PKB); mTOR, mammalian target of rapamycin, a Serine/Threonine protein kinase;; BAX, BCL2-associated X protein; BCL2, B-cell lymphoma protein-2; LC3, microtubule-associated protein light chain 3; IRE1, inositol-requiring protein 1, a serine/threonine protein kinase/endoribonuclease; BiP, binding immunoglobulin protein; JNK2, c-Jun N-terminal protein kinase 2; HSP90, heat shock protein 90; CTRL, control; CLQ, chloroquine; ER, endoplasmic reticulum

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modified in order to improve molecular interactions with specific target- or guest molecules [20].

In the present study, we reveal the mechanisms by which calix[6]arene (CLX6) overcomes the aggressiveness of a human pancreatic cancer cell line (Panc-1). CLX6 dowregulated key protein kinases localized in different cellular compartments resulting in cell cycle arrest, downregulation of pro-survival mediators, endoplasmatic reticulum stress and cell death by autophagy. These broad effects of CLX6 reinforced our hypothesis of its potential as a component of a pharmaceutical formulation for treating pancreatic cancer.

2. Materials and methods

2.1. Reagents

Calix[*n*]arenes ($n = 4, 6$ and 8) and derivatives were provided from Dr Angelo de Fátima (Federal University of Minas Gerais, MG, Brazil). Gemcitabine hydrochloride, 5-Fluorouracil, Propidium iodide (PI), thiazolyl blue tetrazolium blue (MTT) and chloroquine were purchased from Sigma-Aldrich (St. Louis, MO, USA). A Cell Proliferation ELISA, BrdU (colorimetric) kit was obtained from Roche Applied Science (Mannheim, Germany). Reagents for western blotting were purchased from Bio-Rad (Hercules, CA, USA) and Sigma-Aldrich (St. Louis, MO, USA). For western blotting, primary and HRP-conjugated secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA) and Sigma-Aldrich (St. Louis, MO, USA). For confocal microscopy, rabbit polyclonal anti-LC3B was obtained from Cell Signaling Technology (Beverly, MA) and Alexa-456 conjugated secondary antibody was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Transmission electronic microscopy reagents were purchased from Electron Microscopy Sciences (Hatfield, PA, USA) and Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture

Pancreatic cancer cells (Panc-1) were purchased from the Rio de Janeiro Cell Bank (Rio de Janeiro, RJ, Brazil). Panc-1 cells were cultured in Dulbeco's modified Eagle's medium (DMEM) containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (FSB), at 37 °C in an humidified 5% CO_2 atmosphere.

2.3. Treatment of Panc-1 with calix[n]arenes and derivatives

Panc-1 cells (1×10^5 cells/mL) were seeded in a 96-well microplate and after 24 h of culture cells were treated with different concentrations (1, 5, 10, 25, 50 and 100 μM) of calix[*n*]arene and calix[*n*]arene derivatives and incubated for 24 h. Cell viability was analyzed by a MTT reduction assay as described below. Gemcitabine and 5-Fluorouracil (in concentrations up to 2 mM) were used as controls. The control group was treated with the equivalent amount of dimethyl sulphoxide (DMSO, a maximum of 0.1% in the assay mixture was used). Non-treated cell viability was set as 100% and IC_{50} values were determined from three independent experiments.

2.4. Treatment of Panc-1 with CLX6 over time

Panc-1 cells (1×10^5 cells/mL) were seeded in a 96-well microplate and after 24 h the cells were treated with different concentrations (1, 5, 10, 25 and 50 μM) of CLX6. The cells were incubated for 3, 6, 12 and 24 h. Cell viability was analyzed by a MTT reduction assay as described below. Non-treated cell viability at 0 h was set as 100%.

2.5. MTT reduction assay

The treatment medium was removed from the cells after each incubation period. 100 μL of MTT solution (0.5 mg/mL in FBS free culture medium) was added to each well. After incubating for 3 h at 37 °C, the MTT solution was removed and the formed formazan crystals were solubilized in 100 μL of ethanol. The plate was shaken for 10 min and the absorbance was measured at $\lambda = 570$ nm with a microplate reader (Synergy HT, BioTek) [21]. The measured absorbance at $\lambda = 570$ nm was normalized to % of control. This value was calculated by multiplying the absorbance of a treated well by 100 and dividing it by the average absorbance of control wells.

2.6. BrdU incorporation assay

Panc-1 cells (1×10^5 cells/mL) were seeded in a black 96-well microplate and treated with different concentrations (0.1, 1, 5, 10, 25 and 50 μM) of CLX6 for 24 h. The BrdU incorporation assay was performed in accordance with the manufacturer's instructions. In brief, the cells were incubated with BrdU for 3 h. Next the Panc-1 cells were fixated and incubated with anti-BrdU for 90 min. Substrate was added and incubated for 5 min and the chemiluminescence was measured with a microplate reader (Synergy HT, BioTek). The obtained values were normalized to % of control. These values were calculated multiplying the chemiluminescence measurements of treated wells by 100 and dividing them by the average chemiluminescence of control wells, which were considered 100%.

2.7. Flow cytometry for cell-cycle analysis

After treatment with CLX6, cells were spun down, washed with PBS, and resuspended in working solution (1 g/L sodium citrate, 0.5 mg/mL ribonuclease A, 0.05 mg/mL propidium iodide (PI), 0.01% Triton X-100). After incubating in the dark for 60 min at room temperature, the samples were analyzed using a Gallios flow cytometer (Beckman Coulter, USA).

2.8. Western blotting

After treatment with CLX6, Panc-1 cells were lysed in cell lysis buffer (50 mM Tris–HCl (pH 7.4) containing 1% Tween 20, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM o-vanadate, 1 mM sodium fluoride, 1 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mM PMSF) for 2 h. After 10 min of centrifugation, the cleared lysates were immunoprecipitated and resolved by reducing SDS-polyacrylamide gel electrophoresis. The blots were incubated with indicated antibodies and imaged by using chemiluminescence with an ImageQuant LAS 4000 (GE Healthcare Life Science).

2.9. Electron microscopy

Panc-1 cells were incubated in the presence or absence of CLX6 for 24 h and washed in 0.01 M phosphate-buffered saline (PBS), pelleted and subsequently fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 48 h at 4 °C. The cells were postfixed in a solution containing 1% osmium tetroxide and 0.8% potassium ferrocyanide in 0.1 M cacodylate buffer, washed in the same buffer, dehydrated in different concentrations of acetone, and embedded in Epon812 resin. Semithin sections (65 nm) were stained with uranyl acetate and lead citrate. Next, images were made with a transmission electron microscope (JEOL JM 1400).

2.10. Confocal microscopy

Panc-1 cells (4×10^4 cells/mL) were seeded 24 h before treatment on glass coverslips (12 mm diameter) inside the 24-well plates. Cells were treated with 20 μM of CLX6 for 24 h. Next, cells were washed with PBS, fixed for 20 min with 4% p-formaldehyde (PFA) in PBS and quenched with 0.1 M glycin in PBS for 20 min. Then, the cells were permeabilized with 0.2% triton X-100 for 2 min, and blocked with 10% serum solution for 5 min. Subsequently, cells were incubated with

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