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De novo galectin-3 expression influences the response of melanoma cells to isatin-Schiff base copper (II) complex-induced oxidative stimulus

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ABSTRACT

Galectin-3, a ubiquitous member of the galectin family, has been shown to control cellular proliferation, adhesion, migration and apoptosis; thus, it has a role in tumor development and progression. Galectin-3 expression is both up- and down-regulated during melanoma progression. However, conflicting data regarding its roles in tumor biology prompted us to investigate if the presence of galectin-3 influences the response of melanoma cells to a novel metallodrug because metastatic melanoma acquires chemo resistance and is reported to be redox-sensitive. Previously, it was demonstrated that the complex [bis-(2-oxindol-3-yl-imino)-2-(2-aminoethyl) pyridine-N,N′] copper (II) perchlorate, herein referred to as [Cu(isaepy)], induces ROS formation and apoptosis in neuroblastoma cells through mitochondrial uncoupling and the activation of AMPK/p38/p53 signaling. Here, we used a model of vertical growth melanoma (TM1), in which *GAL3* expression is lost during tumor progression. When *de novo* expressed, galectin-3 was found to be ubiquitously present in all subcellular compartments. Our results demonstrate that *de novo* galectin-3 expression impairs the cellular antioxidant system and renders TM1G3 cells more susceptible than *GAL3*-null TM1MNG3 cells to [Cu(isaepy)] treatment. This compound, in contrast with the redox inactive [dichloro (2-oxindol-3-yl-imino)-2-(2-aminoethyl) pyridine-N,N′] zinc (II), herein referred to as [Zn(isaepy)], leads to increased intracellular ROS accumulation, increased carbonyl stress, increased mitochondrial depolarization, decreased cell adhesion, increased p38 activation and apoptosis in TM1G3, compared with TM1MNG3. Cell death was shown to be dependent on a hydrogen peroxide-derived species and on the activation of p38. Because mitochondria are a target of both [Cu(isaepy)] and galectin-3, we propose that the presence of galectin-3 in this organelle favors increased ROS production, thereby inducing oxidative cellular damage and apoptotic death. Therefore, [Cu(isaepy)] may be envisaged as a possible anti-melanoma strategy, particularly for melanomas that express galectin-3.

1. Introduction

Galectin-3 is a ubiquitous member of the galectin family. This lectin is found intracellularly, in the plasma membrane and in the extracellular space [1,2]. Galectin-3 is involved in several processes, such as cell proliferation, adhesion, migration and apoptosis, and is essential for cellular physiology, inflammation, tissue repair and tumor biology. Clinical studies using human tissues have shown that galectin-3 expression is both up-regulated [2–7] and down-[8–11] regulated during tumor progression, including during melanoma development [11–14].

Malignant melanoma is the most aggressive form of skin cancer and accounts for 80% of all skin cancer deaths [15]. Currently approved therapeutic drugs consist of dacarbazine and interleukin-2, which are associated with a poor response. Although some novel targeted therapies are emerging [16,17], including immunotherapy

Abbreviations: [Cu(isaepy)$_2$](ClO$_4$)$_2$, [bis-(2-oxindol-3-yl-imino)-2-(2-aminoethyl) pyridine-N,N'] copper (II) perchlorate; [Zn(isaepy)Cl$_2$], [dichloro (2-oxindol-3-yl-imino)-2-(2-aminoethyl) pyridine-N,N′] zinc (II); AMPK, adenosine monophosphate-activated protein kinase; CAT, catalase; CDNB, 1-chloro-2,4-dinitrobenzene; DAPI, 4′,6-Diamidino-2-phenylindole dihydrochloride; DCFH$_2$-DA, 2′,7′,dichlorodihydro fluorescein diacetate; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); ECM, extracellular matrix; GST, glutathione-S-transferase; HRP, horseradish peroxidase; MTT, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan; Peg, polyethylene glycol; PrPc, cellular prion protein; ROS, reactive oxygen species; SOD, superoxide dismutase.

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and combined drugs, their long term consequences are still unknown [18]. Therefore, novel therapies are clearly required.

Metallodrugs have long been used as antitumor drugs [19]. This class of compounds is particularly interesting as a potential source of novel antimelanoma drugs because melanoma is considered to be very redox-active and sensitive to reactive oxygen species (ROS) [20–22]. The complex produced by copper (II) and disulfiram, for instance, induces apoptosis in melanoma cells [23]. Recently, some metal ligands have been designed [24] based on similarities with indole derivatives (SU9516 or SU6668), which have already been described as inhibitors of the cyclin-dependent kinases [25]. Copper (II) ions, when coordinated with isatin or isatin-derived ligands, induce ROS production, which results in the oxidation of key biomolecules [24,26]. Indeed, the [Cu(isaepy)] complex exhibits anti-neuroblastoma activity by inducing ROS formation, mitochondrial toxicity and apoptosis [24,27–29].

Here, we used a melanoma cell line that lost galectin-3 expression during *in vitro* tumor progression [30,31] and investigated the effect of *de novo* galectin-3 expression following challenge with the [Cu(isaepy)] complex. Our data show that intracellular galectin-3 expression impairs the melanoma antioxidant system and renders cells more susceptible to apoptosis when treated with this copper complex. The mechanisms of cytotoxicity include ROS formation, carbonyl stress, mitochondrial depolarization, p38 activation and cell adhesion inhibition. These processes were most evident in the galectin-3-expressing cells. These results indicate that galectin-3 increases the susceptibility of melanoma cells to apoptosis, and this is most likely to occur through the influence of the protein on the redox effects triggered by [Cu(isaepy)] because the analogous redox inactive [Zn(isaepy)] produced no detectable effect.

2. Material and methods

2.1. Cell lines

Melanoma TM1MNG3 and TM1G3 cells were obtained by stably transfecting TM1 cells [30] with either the pEF1-neo or the pEF1-neo-Gal3 plasmid (kindly donated by Fu-Tong Liu, University of California, Davis), respectively. The cells were routinely cultured in RMPI medium (pH 6.9) (Cultilab, Campinas, Brazil) containing 40 μg/mL gentamicin and 5% fetal calf serum.

2.2. Metal compounds and cell treatment

The copper complex [bis-(2-oxindol-3-yl-imino)-2-(2-aminoethyl) pyridine-N,N'] copper (II) perchlorate, $[Cu(isaepy)_2](ClO_4)_2$, referred to as [Cu(isaepy)], and the analogous zinc complex [dichloro (2-oxindol-3-yl-imino)-2-(2-aminoethyl) pyridine-N,N'] zinc (II), $[Zn(isaepy)Cl_2]$, referred to as [Zn(isaepy)], were synthesized as described [24]. Stock solutions of these complexes (5 mM) were prepared in 10% DMSO and were stored at −20 °C. These solutions were diluted to their final concentrations immediately before the experiments. An aqueous DMSO solution, corresponding to the highest DMSO concentration (0.1%) in the metal solutions, was employed as a vehicle control. Typically, the cells were plated and then treated with the metal complexes for 24 h (or for the specified time) after they had adhered to the plate (usually 4 h later).

2.3. Cell extracts and cell fractionation

Protein extracts and protein cell fractions were prepared as previously described [32].

2.4. SDS–PAGE and immunoblotting

Protein separation and immunoblotting were performed as previously described [33]. The antibodies used were rat monoclonal anti-galectin-3 M3/38 (TIB166, ATCC, 1:10), rabbit anti-p38 and anti-phospho-p38 (Cell Signaling, Boston, USA, 1:1000), anti-rat IgG coupled to HRP (Sigma–Aldrich, São Paulo, Brazil) (1:1000), anti-lamin A (Santa Cruz Biotechnology, Dallas, TX, USA) (1:1000), anti-cytochrome c (Santa Cruz Biotechnology) (1:200), anti-GAPDH (Sigma–Aldrich, São Paulo, Brazil) (1:1000), homemade polyclonal anti-PrPc (1:1000), anti-mouse IgG coupled to HRP (Sigma–Aldrich, São Paulo, Brazil) (1:10,000) and anti-rabbit IgG coupled to HRP (Sigma–Aldrich, São Paulo, Brazil) (1:10,000). The reactions were developed with the West Pico ECL system (Pierce, Rockford, USA).

2.5. Proliferation assay

Cells were plated at a density of 5×10^4 cells/well in 96-wells microplates. Twenty-four hours later, the cells were fixed with 3% paraformaldehyde for 10 min and then incubated with 100 μL of 2% methanol for 10 min before being stained for 10 min with 30 μL of 0.5% crystal violet (Sigma–Aldrich, São Paulo, Brazil) dissolved in 20% methanol (Synth, Diadema, Brazil). The cells were then washed with water, and the dye was extracted with 100 μL of 0.1 M sodium citrate (pH 4.2) containing 50% methanol. The absorbance was read at 570 nm in a microplate reader (BioRad, Hercules, CA, USA).

2.6. Melanin determination

Melanin content was measured as previously described [33]. Briefly, 24 h after the cells were plated, 5×10^5 cells were collected and lysed in 1 M NaOH/10% DMSO. The samples were then boiled for 1 h. The absorbance of the supernatant was determined at 490 nm. The B16F10 and SHSY-5Y cell lines were used as positive and negative controls, respectively.

2.7. Cell adhesion

Ninety-six-wells plates were coated with 15 μg/mL laminin, matrigel (purified according to [34]), vitronectin (purified according to [35]) or fibronectin (purified according to [36]) and incubated for 16 h at 4 °C. The wells were washed and blocked with 2% BSA for 2 h. The cells were treated for 24 h and were then seeded (10^4 cells/well) and incubated for 2 h. Adhered cells were fixed with 3% paraformaldehyde and stained with 0.4% methylene blue in 30% methanol. The dye was extracted with 0.5% acetic acid in 50% methanol. The absorbance was read at 655 nm in a microplate reader (BioRad, Hercules, CA, USA).

2.8. Cell viability

Cells were plated at a density of 5×10^4 cells/well in a 96-wells microplate. Twenty-four hours after treatment with the metal complexes, or with 20 μM menadione (Sigma–Aldrich, São Paulo, Brazil), the cells were washed and incubated with 200 μL of 0.5 mg/mL MTT solution (Sigma–Aldrich, São Paulo, Brazil) for 3 h in a CO_2 incubator. The cells were then washed, and formazan crystals were extracted with 200 μL of DMSO (Sigma–Aldrich, São Paulo, Brazil). The absorbance was read at 570 nm in a microplate reader (BioRad, Hercules, CA, USA). In some assays, the cells were incubated with 25 U/mL Peg-SOD (Sigma–Aldrich, São Paulo, Brazil) and/or 200 U/mL Peg-CAT (Sigma–Aldrich, São Paulo, Brazil) in addition to the metal compounds.