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Cytotoxic effects of mithramycin DIG-MSK can depend on the rise of autophagy

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ABSTRACT

DIG-MSK (demycarosil-3D-β-D-digitoxosyl mithramycin SK; EC-8042), a novel analogue of mithramycin A, induced autophagy in HCT116 human colon carcinoma and, to a lesser extent, in A2780 human ovarian carcinoma cell lines, which was followed by apoptosis and/or necrotic cell death in a time-dependent way. The effects of DIG-MSK included changes in the expression of a set of genes involved in autophagy, the progression of cells through the different phases of cell cycle, and their halting at the checkpoints. Cells treated with the glucose analogue 2-DG (2-deoxy-D-glucose), which induces autophagy because it impairs cell metabolism, or co-treated with 2-DG plus DIG-MSK, also showed altered gene expression and autophagy. In A2780 cells, some genes involved in autophagy were down-regulated by the different treatments, yet the levels of the proteins they encode could be enough to ensure autophagic flux. In HCT116 cells, up-regulation of several pro-autophagic genes resulted in strong autophagic response. Acidic cell organelles and autophagic flux were more evident in HCT116 than in A2780 cells. DIG-MSK was still cytotoxic in cells that underwent autophagy induced by 2-DG. Therefore, we verified that autophagy resulting from a stress response did not protect cells against DIG-MSK, but, instead, autophagy promoted by either 2-DG or the novel mithralogue can enhance the antitumour activity, which depended on the cell type.

1. Introduction

Mithramycin A (MTA) is an aureolic acid polyketide which antitumour activity has been associated with its binding to G/C-rich regions in gene promoters, where it can displace or prevent the binding of transcription factors, chiefly blocking the action of members of the Sp-family of transcription factors (Blume et al., 1991; Fernández-Guizán et al., 2014; Xu et al., 2007). MTA clinical history includes the treatment of Paget's disease (Siris et al., 1980) and of certain cancers such as testicular carcinoma of germinal origin (Brown and Kennedy, 1965), but it fell into disuse because of its toxic side effects. Recently, promising *in vitro* and *in vivo* activities linked to specific modes of action have been described in Ewing sarcoma (Grohar et al., 2011) and lung cancer (Zhang et al., 2012). The new mithramycin analogue demycarosyl-3D-β-D-digitoxosyl-mithramycin SK (DIG-MSK) has been obtained by combinatorial biosynthesis and characterized (Núñez et al., 2012). DIG-MSK shows *in vivo* and *in vitro* antitumor activities similar to that of MTA and other novel analogues, and it is 10-fold less toxic *in vivo* than MTA and 25% less toxic than the structurally related MSK (Núñez et al., 2012). The single maximum tolerated dose of DIG-MSK in mice is the highest among the mithralogues (Núñez et al., 2012). DIG-MSK inhibits the growth of human HCT116 colon carcinoma and A2780 ovarian carcinoma cell lines, where it challenges the interaction between the Sp1 transcription factor and DNA (Vizcaíno et al., 2012, 2014). Furthermore, the *in vivo* evaluation of DIG-MSK antitumor activity by hollow fiber assays and xenograft experiments indicates that it is a promising antitumor drug against several neoplasms (Núñez et al., 2012).

Using A2780 human ovarian carcinoma cells, we have characterized the effects of DIG-MSK on transcription through a genome-wide analysis of changes in gene expression (Vizcaíno et al., 2014). DIG-MSK reduces the expression of a variety of genes, many of which have been related to ovarian cancer progression, but also up-regulates the expression of other genes, consistent with the stress response that chemotherapeutic drugs can produce in treated cells. The effects of DIG-MSK on gene transcription are mainly due to interference with the binding of Sp1 to its putative binding sites in gene promoters, in keeping with our previous

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observations in HCT116 colon carcinoma cells (Vizcaíno et al., 2012). Besides, several biological processes and molecular functions related to transcription and its cellular regulation, including transcription factor activity, were highly influenced by DIG-MSK in A2780 and HCT116 cells (Vizcaíno et al., 2012, 2014).

Macroautophagy (referred to as autophagy hereafter) is an intracellular degradative system that plays central roles in regulating cellular homeostasis, and it is crucial for survival when cells are under metabolic stress (Kroemer et al., 2010; Platini et al., 2010; White and DiPaola, 2009), although whether autophagy causes diseases or protect cells from disease is not clear, as it can promote both cell survival and cell death (Mariño et al., 2014; Notte et al., 2011; Sharma et al., 2014; White and DiPaola, 2009). Autophagy can act as a tumour suppressor mechanism, yet it seems to have different roles in cancer cells in which it would play a complex role in tumour initiation and progression (Gewirtz, 2014b; Notte et al., 2011; White and DiPaola, 2009). On the one hand, autophagy is necessary during the later stages of *in vivo* tumour formation for cancer cell survival in hypoxia conditions before the vascularization of tumour may occur (Kroemer et al., 2010; Yin et al., 2013). On the other hand, autophagy appears to behave as a tumour suppressor in cancer cells, since defective autophagy is associated with malignant transformation and carcinogenesis (Platini et al., 2010; White and DiPaola, 2009). Both A2780 and HCT116 cells bear wild-type p53, which modulates the expression of target genes leading to diverse cellular responses, including cell cycle arrest and apoptosis. Moreover, p53 can transactivate several genes encoding proteins that activate autophagy (Notte et al., 2011). p53 promotes autophagy after genotoxic stress through AMPK (AMP-kinase) activation and mTOR inhibition (Maiuri et al., 2010), although cytoplasmic p53 can also suppress autophagy (Tasdemir et al., 2008).

The glucose analogue 2-deoxy-D-glucose (2-DG) blocks glycolysis, reducing cellular ATP, and it interferes with other cellular processes. 2-DG can be utilized to produce/enhance autophagy in cells (White and DiPaola, 2009), providing us with a tool to study the effects of antitumor drugs in cells undergoing autophagy. Because of the tumour dependence on glycolysis, 2-DG is considered a potential antitumor compound, and it is currently evaluated as an anticancer agent (Maschek et al., 2004; Zhang et al., 2014).

Whether autophagy can cause chemoresistance or it represents a mechanism of cell death after chemotherapy is still a controversial subject (Gewirtz, 2014b; Mansilla et al., 2012; Notte et al., 2011; Radogna et al., 2015; White and DiPaola, 2009), to the point that it cannot be concluded that stress induced autophagy has a protective role against chemotherapy (Gewirtz, 2014a; Mansilla et al., 2015; Sharma et al., 2014). It is necessary to develop successful autophagy-modulating strategies against cancer, together with a better understanding of the roles played by autophagy in the cellular response to chemotherapy and how this could differ depending on the cell type and certain genetic factors. In this way, it is also required to determine how autophagy pathways are activated or inhibited by antitumor agents having different mechanisms of action. For clinical use, it is essential to determine whether enhanced autophagy is a sign of drug responsiveness or resistance (Bincoletto et al., 2013; Notte et al., 2011; Platini et al., 2010).

Here, we explore the crosstalk that may exist between the potent effects of the novel mithramycin analogue DIG-MSK on gene expression (Vizcaíno et al., 2012, 2014) and the presence of pro-autophagic stimuli, and whether this could result in enhanced cytotoxicity or in a protective response against antitumour compounds. We envisage that experimental strategies aimed at activating or inhibiting autophagy could find a wide application for treatments of cancer using novel drug analogues, thus contributing to better clinical results. We show that several genes involved in autophagy were up-regulated while others were down-regulated by the novel mithramycin DIG-MSK in A2780 cells. This was accompanied with a mild autophagic flux. In HCT116 cells, up-regulation of several of those genes resulted in a stronger autophagic response. In the presence of 2-DG, which impairs cell metabolism and induces autophagy, DIG-MSK was still active. Therefore, it was verified that autophagy resulting from a stress response had no cytoprotective function against DIG-MSK, but it would even enhance the antitumour activity, which was more evident in HCT116 cells.

2. Materials and methods

2.1. Cell culture and drug treatments

HCT116 human colon carcinoma cells were growth in 50% DMEM (Life Technologies, Alcobendas, Spain)/50% Ham's F12 (Lonza, Barcelona, Spain) medium. A2780 human ovarian carcinoma cells were grown in RPMI 1640 medium (Life Technologies) supplemented with 2 mM sodium pyruvate. Both culture media were supplemented with 10% foetal bovine serum (Life Technologies), 100 U/ml penicillin (Life Technologies), 100 μg/ml streptomycin (Life Technologies), and cells were allowed to grow at 37 °C in a humidified atmosphere with 5% CO_2.

DIG-MSK (demycarosil-3D-β-D-digitoxosyl mithramycin SK) was isolated and purified (HPLC ⩾ 97%) from the producer *Streptomyces* species as described elsewhere (Núñez et al., 2012). Stocks of DIG-MSK were prepared as 1 mM solutions in sterile 150 mM NaCl, maintained at −20 °C, and brought to the final concentrations just before use. Exponentially growing A2780 or HCT116 cells subculturated at a density of 2.5×10^4 cells/ml were treated for different periods of time with DIG-MSK, 2-DG or 2-DG plus DIG-MSK at the concentrations specified in the legends to figures. Those DIG-MSK concentrations, in the nanomolar range, used to treat cells were determined elsewhere (Vizcaíno et al., 2012, 2014).

2.2. Cytometric assessment of apoptosis and necrosis

Exponentially growing A2780 or HCT116 cells were grown in the presence of 2-DG, DIG-MSK or 2-DG plus DIG-MSK, for different periods of time (see the legends to figures). Primary apoptosis was determined by flow cytometry as the Annexin-V-fluorescein positive/PI negative cell population by using the Annexin-V-Fluos staining kit (Roche Diagnostics) and flow cytometry in a Coulter Epics-XL flow cytometer (Beckman Coulter, Hialeah, FL). A cellular gate was established on forward scatter (FS) vs. side scatter (SS) dotplots, which included both live and dead cells, while aggregates, small fragments, and apoptotic bodies were excluded by this gate. Necrotic cells were characterized as two populations: Annexin-V-Fluos negative/PI positive (primary necrotic cells, by loss of membrane ability to exclude PI) and Annexin-V-Fluos positive/PI positive (necrosis arising from apoptotic cells or primary necrosis).

2.3. Detection of acidic vesicular organelles (AVOs) by flow-cytometry and fluorescence microscopy

For flow cytometry, A2780 and HCT116 cells at a density of 2.5×10^4 cells/ml were grown in 25 cm^2 flasks (Corning, Cultek, Madrid, Spain), and treated with 2-DG, DIG-MSK or 2-DG plus DIG-MSK for 24 h. Cells were harvested and resuspended in PBS, incubated with 5 μg/ml acridine orange (Sigma–Aldrich, St. Louis, MO) for 15 min at room temperature, washed twice with PBS, and analyzed in a Gallios flow cytometer (Beckman Coulter, Miami, FL). Red (650 nm) fluorescence emission from 10.000 cells

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