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.

Keywords: Autophagy; mithramycin; cell death; A2780 cells; HCT116 cells

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.,
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 mitralogues (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; Vizcaíno et al., 2014). Furthermore, the in vivo evaluation of DIG-MSK antitumor activity by hollow fibre 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 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; Vizcaíno et al., 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; Vizcaíno et al., 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 grown 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% CO₂.

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 subcultured at a density of 2.5 x 10⁴ 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; Vizcaíno et al., 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 x 10^4 cells/ml were grown
in 25 cm² flasks (Corning, Cultek, Madrid, Spain), and treated with 2-DG, DIG-MSK or 2-DG
plus DIG-MSK for 24 hours. Cells were harvested and resuspended in PBS, incubated with 5
µg/ml acridine orange (Sigma-Aldrich, St. Louis, MO) for 15 minutes at room temperature,
washed twice with PBS, and analysed in a Gallios flow cytometer (Beckman Coulter, Miami,
FL). Red (650 nm) fluorescence emission from 10.000 cells illuminated with blue (488 nm)
excitation light was measured. Fluorescence intensity values were used to calculate the relative
fluorescence ratio (treated vs. control cells).

For fluorescence microscopy, cells plated on coverslips were treated with 2-DG, DIG-MSK
or co-treated with 2-DG plus DIG-MSK for 24 h before 1µg/ml acridine orange was added for
15 min at room temperature. DAPI (Sigma-Aldrich) was also added (8 µg/ml) to cells as a
nuclear counterstain. Cells were washed twice with PBS and, after adding fresh media,
fluorescent micrographs were taken using a Nikon E1000 Microscope (Nikon Instruments,
Amstelveen, Nederland). Images of fluorescent cells were captured using a CoolSNAP camera
(Photometrics, Tucson, AZ) and analysed using ImageJ software (Schneider et al., 2012).

2.4. Immunoblot analysis

Protein was extracted from untreated HCT116 or A2780 cells and from cells treated with 2-
DG, DIG-MSK, or 2-DG plus DIG-MSK, by using a lysis buffer consisting of 50 mM Tris–HCl
(pH 8.0), 150 mM NaCl, 5 mM EDTA, 0.5% Igepal (NP-40) and 0.1 mM phenylmethylsulfonyl fluoride, containing 2 μg/ml aprotinin (Sigma-Aldrich) and 1 μg/ml leupeptin (Sigma-Aldrich). Total protein was quantified by the Bradford assay (Bio-Rad, Hercules, CA). About 50 μg of denatured protein was subjected to electrophoresis on SDS-polyacrylamide gels, blotted onto Optitran BA-S85 membranes (Schleicher & Schuell, Dassel, Germany), probed with the specific antibodies for LC3 (MBL, BioNova, Madrid, Spain), Beclin 1 (AbDSerotec, BioNova), Anti-p62/SQSTM1 (Sigma-Aldrich), and β-tubulin (Merck Millipore, Madrid, Spain), incubated with secondary antibodies (Jackson ImmunoResearch, Newmarket, UK) and detected by chemiluminescence using the Immobilon Western HRP Substrate (Millipore). Autophagic flux was measured in the presence of 100 nM BafA1 (bafilomycin A1, Sigma-Aldrich).

2.5. RNA extraction and quantitative real-time PCR analysis

Total RNA was extracted from control (untreated) cells and from cells treated with 2-DG, DIG-MSK, or co-treatments with 2-DG plus DIG-MSK for 24 h. The UltraspecRNA isolation reagent (Biotecx, Houston, TX) was used following the procedure provided by the supplier. RNA was digested with RNAse-free DNase I (Roche Diagnostics, Madrid, Spain) in the presence of RNAse inhibitors (RNasin, Promega Biotech Iberica, Madrid, Spain), phenol extracted and precipitated, and the pellet was dissolved in RNAse-free water. The yield and purity of total RNA were assessed spectrophotometrically and RNA integrity examined in an Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE).

For quantitative real time PCR (qRT-PCR), cDNAs were synthesized from 2 μg of isolated RNA obtained from two biological replicates, in a 20 μl reaction volume using the Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics) following manufacturer's instructions. A set of ten human genes involved in the response to cellular stress, autophagy and apoptosis were analyzed by qRT-PCR. Namely: Beclin1, ATG3, ATG4B, NFKB1, LC3, BCL2 (Bcl-2), p62/SQSTM1, p53, p21WAF1 and Bax, as well as GAPDH, which was used as internal
The primers used for qRT-PCR are listed in Table 1. Reactions were performed in triplicate using the SYBR-Green PCR Master Mix (Roche Diagnostics). Amplification and detection were performed in triplicate in a Roche LightCycler 480 system. PCR conditions included an initial denaturation step at 95 °C for 10 min, followed by 45 cycles of a denaturation step at 95 °C for 10 s, an annealing step at 60 °C for 30 s, and an extension step at 72°C for 10 s. Series of 10-fold dilutions of cDNA were used to generate the standard curves to calculate the efficiency of the amplifications. Reactions in absence of template RNA and in the absence of enzyme were also performed for each primer pair as negative controls. A final dissociation curve was generated to verify that a single product was amplified. Relative expression values of the different genes were calculated from the threshold cycle (Ct) following the \( \Delta \Delta \text{Ct} \) method (Pfaffl, 2001), using GAPDH as an internal housekeeping control.

2.6. Statistical analysis

Statistical differences were determined using SPSS software v.21 (IBM Corp., Armonk, NY). The data were expressed as the mean ± SD. Comparisons were performed using one-way ANOVA followed by Tukey’s post-hoc test.

3. Results

3.1. The novel mithramycin DIG-MSK and the glucose analogue 2-DG induce the formation of acidic vesicular organelles (AVOs) in A2780 and HCT116 cells

A2780 human ovarian carcinoma cells treated with DIG-MSK or 2-DG for 24-h, as well as those co-treated with both compounds for 24 h (at the concentrations indicated in the legend to Fig. 1), showed the formation of AVOs (Fig. 1). In Fig. 1A, the upper panels show the quantification of AVOs in A2780 cells by flow-cytometry after acridine orange staining of treated cells and control, untreated, cells. Production of AVOs in acridine orange-stained A2780 cells was modest, yet it was confirmed by fluorescence microscopy (bottom panels in Fig. 1A). Although induction of AVOs by either DIG-MSK or 2-DG, or by the co-treatment with both
molecules, was moderate in these cells, AVOs fairly accumulated in co-treatments. The results of
the equivalent experiments undertaken in HCT116 cells are shown in Fig. 1B. Altogether, the
quantification of AVOs by flow cytometry and the direct observation of intracellular AVOs by
fluorescence microscopy indicated that autophagy induced by the different treatments was higher
in HCT116 cells than in A2780 cells (cf. upper panels in Fig. 1A and 1B).

The relationship between induction of AVOs and autophagy in either cell line upon
treatments was further evidenced by detecting the presence of autophagic flux (see below). Given
that DIG-MSK is a potent antitumor agent, we first studied whether the induction of AVOs may
be associated with cell death.

3.2. The glucose analogue 2-DG can potentiate both apoptosis and necrotic cell death induced by
mithramycin DIG-MSK

The glucose analogue 2-DG was used to analyse whether activating autophagy pathways in
A2780 and HCT116 cells may either enhance or reduce the induction of apoptotic cell death. We
analysed whether autophagy may result in alterations in the cytotoxicity of mithramycin DIG-
MSK, and if cell death followed apoptotic or necrotic pathways. A time-course analysis was
performed to examine whether DIG-MSK and 2-DG produced apoptosis/necrosis alone and if co-
treatment with both compounds may increase the apoptotic cell death (Fig. 2). In A2780 cells, co-
treatment with 2-DG plus DIG-MSK induced low percentages of cell death after 5 h (6.5%) and
24 h (2.9%), but cell death increased up to 15.6 % in 72-h treatments (Fig. 2A). Dead cells scored
as both necrotic and apoptotic cells, based on their staining by Annexin-V-Fluos or propidium
iodide, yet they were mainly observed as secondary apoptotic/necrotic cells (double staining)
after 72-h treatments, which indicated those cells were first dying by either necrosis/necroptosis
or apoptosis (Fig. 2A). The co-treatment of A2780 cells with DIG-MSK plus 2-DG for 5 h
increased the percentage of cell death by apoptosis, while DIG-MSK alone produced 5.6%
primary apoptosis after 24-h treatments (Fig. 2A). Those results were consistent with that the
presence of AVOs did not protect A2780 ovarian cancer cells from the cytotoxic effects of DIG-MSK.

The time-dependent effects of either DIG-MSK, 2-DG, or the co-treatments, were more pronounced on HCT116 cells than in A2780 cells (Fig. 2). Primary apoptosis reached 6.1% in 24-h co-treatments with 2-DG plus DIG-MSK, which the highest value among all the experimental conditions displayed in Fig. 2B. This was accompanied by a 38.7% secondary apoptosis/necrosis. HCT116 cells were more sensitive than A2780 cells to treatment with the glucose analogue (cf. the 24-h experiments in Figs. 2A and 2B). Besides, DIG-MSK was inducing apoptosis after 72-h treatments (26.3% primary apoptosis, with 23.3% viable cells), whereas in A2780 cells the percentage of cells undergoing primary apoptotic death was only 5.6% after 24 h. Of note, in 5-h or 24-h treatments the presence of AVOs (Fig. 1) did not prevent the cytotoxicity of DIG-MSK, but it could enhance it (Fig. 2), although it does not appear to be the case after 72 h. The strong inhibition attained in HCT116 cells after 24-h co-treatments (Fig. 2B) coincided with a higher number of AVOs (Fig. 1B), which, in turn, might be related to autophagy.

3.3. Immunobloting analyses revealed that DIG-MSK induced time-dependent autophagy in A2780 and HCT116 cells

We sought further insights into whether the effects of either DIG-MSK, 2-DG, or co-treatments with DIG-MSK plus 2-DG were linked to the presence of autophagy. Both cytometry and fluorescence microscope, described above (Fig. 1), showed the presence of AVOs, which suggested the induction of autophagy in HCT116 cells and, to a lesser extent, in A2780 cells, yet they did not prove it. Therefore, we assessed by immunoblotting the levels of LC3 protein and the presence of the lipidated form LC3-II, as well as the intensification of autophagic flux, as reliable molecular markers of autophagy (Mizushima et al., 2010). Beclin 1 and p62/SQSTM1 (sequestosome 1) were also evaluated. Figure 3 shows the changes induced by the different treatments in the levels of p62 and Beclin 1 proteins, and on the LC3 turnover in both A2780 and
HCT116 cells. All the experiments were performed in the presence of BafA1 (bafilomycin A1), which prevents maturation of autophagic vacuoles, to detect the existence of autophagic flux. Conversion of the cytosolic LC3-I protein into the LC3-II lipidated form was detected in A2780 cells after the different treatments (Fig. 3A). Accumulation of Beclin 1 and LC3 turnover were consistent with both that autophagy was induced by 2-DG and that autophagic flux accompanied the DIG-MSK antitumour activity. Interestingly, the Beclin 1 levels were lower in co-treated A2780 cells after 5 h and 24 h (Fig. 3A), which would facilitate apoptosis (Kang et al., 2011) that was somewhat enhanced at 24 h (Fig. 2A). In general, the levels of p62 remained almost unaltered, although some additional accumulation of this protein occurred after co-treatments (Fig. 3A).

In HCT116 cells, accumulation of the LC3-II form, determined in the presence of BafA1, indicated that 5-h treatments with DIG-MSK induced autophagy, even in the absence of induction by 2-DG. As expected, 2-DG alone also induced autophagy (Fig. 3B). The levels of p62 decreased slightly after 5-h treatments, but, on the contrary, they remained high in 24-h treatments, while autophagy was still occurring (as deemed by the LC3 turnover (Fig. 3B)). Those maintained, or slightly increased, p62 levels in 24-h treatments were at variance with the expected autophagic degradation of p62/SQSTM1. Nevertheless, because p62 function is not limited to autophagy (Moscat and Diaz-Meco, 2012), the present results may reflect alternative roles of this protein in treated cells that maintain its high levels (see section 4.). Besides, Beclin1 was detected when LC3-II was observed upon treatments, which indicated that autophagy was induced (Fig. 3B). Altogether, in this cell line, the increase in autophagy was time-dependent, occurred upon treatment with DIG-MSK, and it was somewhat eased by the presence of 2-DG.

3.4. DIG-MSK changes the expression of genes involved in the autophagy and apoptotic pathways
DIG-MSK is a DNA-binding drug known to alter the transcriptome of A2780 and HCT116 cells (Vizcaíno et al., 2012; Vizcaíno et al., 2014). We sought to unveil whether autophagy may correlate with changes in gene expression, which ultimately may affect the levels of apoptosis and the increase in necrotic/necroptotic death described above. For each cell line, changes induced by the different treatments in the expression of a set of selected genes were quantified by qRT-PCR. In general, changes in gene expression depended on both the cell line and the treatments (Fig. 4). Both up- and down-regulated genes were detected and quantified. Most of the differences in gene expression were statistically significant according to one-way analysis of variance (ANOVA) followed by a post-hoc test (see section 2., and the legend to Fig. 4).

In A2780 cells, DIG-MSK up-regulated the expression of four of the ten genes analysed by qRT-PCR (Beclin 1, LC3, and p21WAF1 (p < 0.01) and p53 (p = 0.07)) while it down-regulated five genes significantly (ATG3, NFKB1, Bcl-2, p62 and Bax) (Fig. 4A). The changes in gene expression induced by DIG-MSK in HCT116 cells (Fig. 4B) were distinctly different from those occurring in A2780 cells. Six genes were significantly (p < 0.01) up-regulated (Beclin 1, ATG3, NFKB1, Bcl-2, p53 and p21WAF1), while three (LC3, p62 and Bax) were down-regulated (p < 0.01) (Fig. 4B).

Up-regulation of p21, LC3 and p53 in A2780 cells was consistent with both a transient halt of cells in the cell cycle checkpoints, seemingly to facilitate damage repair, and autophagy, while reducing Bcl-2 expression could be needed for preventing the blocking by Bcl-2 of cell death induced by the mithramycin analogue. At first glance, some of the results obtained in HCT116 cells seemed rather outlandish since Bcl-2 was up-regulated, while Bax was down-regulated, a situation that could argue against an apoptotic response. Nevertheless, we detected apoptotic cell death unambiguously, although it was accompanied by necrosis/necroptosis and secondary necrosis (Fig. 2B). Abrogation of LC3 expression in HCT116 cells (Fig. 4B) was at variance with the rise of autophagy. The down-regulating transcriptional effect could be counterbalanced by the
presence of enough LC3 protein, seemingly LC3-II, in the cytoplasm as detected by western blot (Fig. 3B). Moreover, the enhanced expression of Beclin1 and ATG3, and the down-regulation of p62 after treatments with DIG-MSK (Fig. 4B), were fully consistent with the rise of autophagy.

3.5. Co-treatment of A2780 and HCT116 cells with 2-DG plus DIG-MSK alters the expression of genes related to autophagy and apoptosis

Figure 4 shows the effects produced by the co-treatments with 2-DG plus DIG-MSK on gene transcription. Five genes (Beclin 1, LC3, p53, p21WAF1 and Bax) were significantly up-regulated in A2780 cells (Fig. 4A). In these cells, the up-regulation of Beclin 1 and LC3 was in keeping with the upsurge in autophagy (Fig. 3A). In HCT116 cells, the co-treatment produced the significant (p < 0.01) up-regulation of NFkB and two genes (ATG3, ATG4B) directly involved in autophagy, as well as of genes implicated in halting cells at the checkpoints and in apoptosis (p53, p21WAF and Bcl-2). Besides, the expression of LC3 was down-regulated in HCT116 cells by the co-treatment with 2-DG plus DIG-MSK (Fig. 4B). Given that autophagy was still functional during the treatments, it seems the cells contained sufficient cytoplasmic LC3 protein (Fig. 3), which would make the down-regulation of its coding gene a minor handicap for the observed short-time (fast) autophagic response. The up-regulation of Bcl-2 was in line with the occurrence of apoptotic cell death following autophagy.

4. Discussion

In this study, we investigated a DNA-binding antitumour agent, the mithralogue DIG-MSK, which can induce autophagy while still committing cells to die by apoptosis/necrosis, thus autophagy precedes and might facilitate drug-induced cell death. DIG-MSK produces autophagy in A2780 human ovarian cancer cells and HCT116 human colon carcinoma cells regardless of the presence of the glucose analogue 2-DG.
Whereas cell death pathways and autophagy share some features and they are both activated by the new mithramycin DIG-MSK, and the glucose analogue 2-DG, there are clear differences in how this occurs in A2780 and HCT116 cells. Autophagy, which is higher in HCT116 cells than in A2780 cells (Figs. 1 and 3) is documented by the presence of AVOs (Fig. 1), the upsurge in active LC3-II protein, and the detection of autophagic flux (Fig. 3A). In A2780 cells, DIG-MSK produces more autophagy than 2-DG (Figs. 1A and 3A), while co-treatments for 5 h or 24 h, do not seem to result in an enhancement of autophagy (Fig. 3A). In HCT116 cells, 5-h treatments with DIG-MSK induced autophagy, while co-treatments for 5 h or 24 h resulted in a slightly lower autophagy (Fig. 3B).

LC3-II usually binds to p62/SQSTM1 to regulate protein delivery to autophagosomes (Bincoletto et al., 2013). Strikingly, p62 was observed in both cell lines upon treatments (Fig. 3). The levels of p62 in HCT116 cells were only slightly reduced by the different treatments after 5 h, as well as in 24-h treatments with DIG-MSK. However, they were up-regulated in the other 24-h treatments, regardless of the autophagic flux (Fig. 3B). In both cell lines, high p62 protein levels were in conflict with the expected autophagic degradation of p62 (Pankiv et al., 2007). Our experimental data suggest that p62 could be involved in cell pathways other than autophagy in A2780 and HCT116 cell lines, thus it was protected from being degraded (Moscat and Diaz-Meco, 2012). Although p62 levels were not usable for estimating autophagic flux, autophagy was still measurable through the LC3-II levels determined in the presence of BafA1 (Fig. 3B). It is worth noting here that dysregulation of p62, which could encompass the control of its degradation, is implicated in Paget’s disease (Layfield et al., 2006), a pathology that has been clinically treated with MTA (Siris et al., 1980). Given that DIG-MSK could modify the activity of p62, maintaining its cytoplasmic levels even when autophagy is active, we foresee that the more active analogue DIG-MSK might be an advantageous alternative in the treatment of Paget’s disease.
Apart from the onset of autophagy with the different treatments, we have observed time-dependent cell death in both cell lines with co-treatments (Fig. 2). DIG-MSK is more cytotoxic in HCT116 cells than in the A2780 cells (Fig. 2), in agreement with our previous observations (Vizcaíno et al., 2012; Vizcaíno et al., 2014). Our results provide insights into how DNA-binding agents, which have been described as transcription inhibitors by interfering with the interaction of transcription factors with DNA, can produce the development of autophagic response in cancer cells bearing wild-type p53, a singularity described to occur in cells containing inactive p53 protein (Mansilla et al., 2015; Notte et al., 2011; White and DiPaola, 2009). Our results are consistent with p53 participating in autophagy by mechanisms that are likely to differ among different cell types (Maiuri et al., 2010; Scherz-Shouval et al., 2010), and, as well, with autophagy having a cytotoxic function (Gewirtz, 2014a; Mansilla et al., 2015; Sharma et al., 2014). In HCT116 cells, in which we observed the higher autophagic response to treatments, it has been reported that p53 down-regulates LC3 RNA and protein levels, which forces maintaining autophagic flux over longer periods of time (Scherz-Shouval et al., 2010).

While analysing transcription is justified by the “anti-transcriptional” activity of DIG-MSK and other DNA-binding drugs, it should be remarked here that post-translational modification of autophagy proteins has emerged as an essential regulatory mechanism in response to nutrient availability and growth factor deprivation (Wani et al., 2015). In this context, some of the changes observed in gene expression may involve a response to cellular stress rather than direct inhibition of protein-DNA interactions. Stress-responsive transcription factors such as p53 and NFκB, which are encoded by genes whose expression is altered by DIG-MSK (Fig. 4), play key roles in the regulation of the autophagic response (Mariño et al., 2014; Pietrocola et al., 2013). Whilst in the nuclei some p53-inducible genes undertake pro-autophagic functions, the cytoplasmic p53 can inhibit autophagy (Tasdemir et al., 2008). NFκB and autophagy are deeply linked through a complex network of both transcriptional and transcriptional-independent signals.
(Pietrocola et al., 2013), and activation of NFkB could be required for optimal autophagic response (Criollo et al., 2012).

At odds with several studies that have shown that tumour resistance to antitumour agents is often associated with the up-regulation of autophagy (White and DiPaola, 2009), our results agree, however, with the growing evidence that autophagy induction can also enhance the activity of antitumour agents (Gewirtz, 2014b; Mansilla et al., 2015; Notte et al., 2011), and it is even the case that reducing autophagy can be a mechanism for acquiring resistance to chemotherapy (Sirichanchuen et al., 2012). Transcriptional changes in A2780 cells could concur with a transient halt of cells in the cell cycle checkpoints (Vizcaíno et al., 2014), which would facilitate damage repair and the rise of autophagy. The situation seems more intricate in HCT116 in which enhanced Bcl-2 expression and low Bax expression levels (Fig. 4B) would argue against apoptotic death, yet cells can still be committed to dying through necrosis/necroptosis (Fig. 2B). Given that the Bcl-2 family of proteins not only regulates apoptosis but also participates in controlling autophagy (Levine et al., 2008), it is of little wonder that autophagy could facilitate rather than inhibit cell death (Fig. 2). Down-regulation of LC3 in HCT116 cells after treatments was also staggering, although in the onset of autophagy it is conceivable that enough cytoplasmic LC3 protein could be present, and its conversion into the lipidated LC3-II form would explain the enduring autophagic flux (Fig. 3B). In line with the induction of autophagy upon treatments, we also detected up-regulation of Beclin 1, which is up-regulated during cellular stress (Kang et al., 2011), and of ATG3 and ATG4B, two genes responsible for protein transport, targeting to membranes and protein degradation (Krick et al., 2008).

The metabolic stress induced by 2-DG can be useful not only for cancer prevention and treatment (White and DiPaola, 2009), but also as an advantageous modulator of antitumour chemotherapy through the induction of autophagy (Levy and Thorburn, 2011; Notte et al., 2011).
Our observations suggest the interest of confirming the enhanced antitumour effects of the in vivo combination of DIG-MSK and 2-DG.

Conflict of interests

L-EN and and FM are employees of EntreChem SL, FM reports ownership of stock in EntreChem SL. All other authors declare they have no conflict of interests.

Acknowledgements

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References


LEGENDS TO FIGURES

Fig. 1. Formation of acidic vesicular organelles (AVOs). (A) Formation of AVOs in A2780 human ovarian carcinoma cells treated with 80 nM DIG-MSK, 4 mM 2-DG or co-treated with 4 mM 2-DG plus 80 nM DIG-MSK for 24 h, quantified by flow cytometry after acridine orange staining (upper panels; red: control, blue: treated cells) and examined by fluorescence microscopy (bottom panels). Red fluorescence (λem = 650 nm) vesicles are AVOs. (B) Formation of AVOs in HCT116 human colon carcinoma cells treated with 11 nM DIG-MSK, 6.4 mM 2-DG or co-treated with 6.4 mM 2-DG plus 11 nM DIG-MSK, quantified by flow cytometry after acridine orange staining (upper panels red: control, blue: treated cells) and examined by fluorescence microscopy (bottom panels). Red fluorescence (λem = 650 nm) vesicles are AVOs. Bars inside the micrographs correspond to 200 µm.

Fig. 2. Detection of cell death by apoptosis and/or necrosis. (A) A2780 cells treated with 80 nM DIG-MSK, 4 mM 2-DG or co-treated with 4 mM 2-DG plus 80 nM DIG-MSK for 5, 24 and 72 h. Adherent (attached) and detached (floating) cell populations were pooled together and stained with Annexin-V-Fluos and PI (propidium iodide), and analysed by flow cytometry. Quantification of the cell populations is shown under the different panels: A, (Annexin-V-Fluos positive/PI negative) apoptotic cells; N, (Annexin-V-Fluos negative/PI positive) necrotic cells; SA+SN, (Annexin-V-Fluos positive/PI positive) secondary apoptotic/necrotic cells; V, viable cells. (B) HCT116 cells treated with 11 nM DIG-MSK, 6.4 mM 2-DG or co-treated with 6.4 mM 2-DG plus 11 nM DIG-MSK for 5, 24 and 72 h. Other details as in panel A.

Fig. 3. Western blot analysis of the time-dependent effects of 2-DG and DIG-MSK on autophagic activity. The levels of p62/SQSTM1, Beclin 1, LC3-I and LC3-II proteins were detected by immunoblotting in experiments undertaken in the presence of 100 nM bafilomycin
A1 (BafA1) to evaluate autophagic flux. (A) Immunoblots of total protein extracted from A2780 untreated cells, and from cells treated with 80 nM DIG-MSK, 4 mM 2-DG or co-treated with 4 mM 2-DG plus 80 nM DIG-MSK for 5 h and 24h. β-tubulin (Tub) blot was used as a protein loading control. The LC3-II/Tub ratio is shown to compare the normalized amount of LC3-II among samples as an indicator of autophagy. (B) Immunoblots of total protein extracted from untreated cells, and from HCT116 cells treated with 11 nM DIG-MSK, 6.4 mM 2-DG or co-treated with 6.4 mM 2-DG plus 11 nM DIG-MSK for 5 h and 24h. β-tubulin (Tub) blot was used as a protein loading control. The LC3-II/Tub ratio is shown to compare the normalized amount of LC3-II among samples as an indicator of autophagy. The figure shows a representative experiment of experiments performed in duplicate with similar results.

Fig. 4. Gene expression in A2780 and HCT116 cells. (A) Relative gene expression in A2780 cells of a set of endogenous genes after treatments with 80 nM DIG-MSK, 4 mM 2-DG or co-treatments with 4 mM 2-DG plus 80 nM DIG-MSK for 24h. Gene expression was quantified by qRT-PCR, normalized using the housekeeping GAPDH gene, and compared to gene expression in untreated cells. Data are mean ± SD from three independent experiments. (B) Relative gene expression in HCT116 cells of a set of endogenous genes after treatments with 11 nM DIG-MSK, 4 mM 2-DG or co-treatment with 6.4 mM 2-DG plus 11 nM DIG-MSK for 24 h. Other details as in panel A. For all the genes, a statistical analysis was performed to evaluate the differences in gene expression. In both cell lines, there was a statistically significant difference among the different groups (treatments) for all the genes analysed (p values < 1E-4, except for ATG4B in A2780 cells: p < 4E-3), as determined by one-way ANOVA. A Tukey’s post-hoc test was undertaken for two-sample comparisons between treatments. For the sake of clarity, the figure only depicts statistical differences between control, untreated, cells and both the treatments with DIG-MSK alone and co-treatments with DIG-MSK plus 2-DG (**p < 0.01, *p <
0.05), as adding all the comparisons in the figure would result in a troublesome picture. See main text for additional details.
Figure 1
Figure 2
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Figure 3
Figure 4