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Inhibition of MCL-1 by obatoclax sensitizes Sp2/0-Ag14 hybridoma cells to glutamine deprivation-induced apoptosis

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For several cancer cell types, the lack of an adequate supply of the amino acid L-glutamine (Gln) triggers apoptosis, a phenomenon termed Gln addiction. In this report, we examined the role of the anti-apoptotic proteins of the B-cell lymphoma 2 (BCL-2) protein family in the survival of Sp2/0-Ag14 (Sp2/0) mouse hybridoma cells, a cell line that undergoes apoptosis within minutes of Gln deprivation. Western blot analysis revealed that myeloid cell leukaemia 1 (MCL-1) was expressed at much higher levels than BCL-2, B-cell lymphoma extra-large and BCL-2-like protein 2 making it the prominent pro-survival BCL-2 family member in this hybridoma. Gln deprivation triggered a progressive decrease in MCL-1 protein levels, which coincided with the decrease in Sp2/0 cell survival. Moreover, Sp2/0 cells were much more sensitive to the broad Bcl-2 homology domain-3 (BH3) mimetic obatoclax (which targets MCL-1) than to the more selective drug ABT-737 (which does not target MCL-1). Finally, we show that obatoclax sensitizes Sp2/0 cells to apoptosis following Gln starvation. All together, the data presented here reveal that modulation of the pro-survival protein MCL-1 is an important step in the sequence of events leading to the initiation of apoptosis in Gln-starved Sp2/0 cells. Cancer cells require an adequate supply of L-glutamine for their survival. Using a mouse hybridoma cell line that is exquisitely sensitive to glutamine starvation, we show that the levels of the pro-survival BCL-2 family protein MCL-1 decrease upon glutamine starvation in a manner that correlates with the loss of cell viability. Moreover, inhibiting MCL-1 with the drug obatoclax sensitizes hybridoma cells to glutamine starvation. Thus, in some cancer cells, glutamine starvation triggers the inactivation of pro-survival proteins. Our data suggest that the combined inhibition of glutamine biosynthesis pathways and BCL-2 proteins may prove effective against some cancers. Copyright © 2015 John Wiley & Sons, Ltd.

KEY WORDS—apoptosis; glutamine; glutamine addiction; hybridoma; MCL-1; nutrient starvation; obatoclax

LIST OF ABBREVIATIONS—BAD, Bcl-2-associated death promoter; BAK, BCL-2 homologous antagonist killer; BAX, B-cell lymphoma associated X; BCL-2, B-cell lymphoma 2; BCL-X_L, B-cell lymphoma extra-large; BCL-W, BCL-2-like protein 2; BH3, BCL-2 homology domain 3; BID, BH3-interacting domain death agonist; BIM, Bcl2-interacting mediator of cell death; BSA, Bovine serum albumin; FCS, FetalClone I; Gln, L-glutamine; IMDM, Iscove's modified Dulbecco's medium; MCL-1, Myeloid cell leukaemia 1; MOMP, mitochondrial outer membrane permeabilization; mTOR, Mechanistic target of rapamycin; PUMA, p53 upregulated modulator of apoptosis; SMAC/Diablo, Second mitochondrial-derived activator of caspase / Direct IAP-binding protein with low pI; Sp2/0, Sp2/0-Ag14; TTBS, Tris-buffered saline/Tween 20; XIAP, X-linked inhibitor of apoptosis protein

INTRODUCTION

The amino acid L-glutamine (Gln) is an important component of intermediate metabolism, where it acts as a precursor for protein and nucleotide synthesis and as cellular fuel.[1,2] The contribution of Gln to metabolism is especially important for actively proliferating cells, where the amino acid is used to replenish tricarboxylic acid cycle intermediates and to generate the reduced form of nicotinamide adenine dinucleotide phosphate, a reducing co-factor essential to many biosynthetic reactions.[3] Furthermore, Gln and its metabolites modulate several signalling events promoting cell proliferation and survival.[2] As a consequence, cancer cells show an increased dependence toward this amino acid, a phenomenon called Gln addiction.[4] While the Gln requirement of cancer cells has been known since the 1950s,[5] the precise cellular and molecular events linking Gln to such a complex phenotype as cell survival need to be studied in more detail.[6]

In several normal or transformed cell types, Gln deprivation triggers a stress response that leads to death by apoptosis.[7] In particular, several studies have shown that apoptosis triggered by Gln starvation can be inhibited by pro-survival members of the B-cell lymphoma 2 (BCL-2) family of proteins.[8–10] These proteins, which include BCL-2, B-cell lymphoma extra-large, myeloid cell leukaemia 1 (MCL-1) and BCL-2-like protein 2 are well known for their role in the modulation of the intrinsic apoptotic pathway.[11,12] Mitochondrial outer membrane

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permeabilization (MOMP) is the central event in this pathway and is triggered by the formation of pores by B-cell lymphoma associated X and BCL-2 homologous antagonist killer, two pro-apoptotic proteins of the BCL-2 family.[12] The BAX/BAK protein pores cause the release of apoptogenic factors such as cytochrome *c* and Second mitochondrial-derived activator of caspase / Direct IAP-binding protein with low pI from the mitochondrial intermembrane space into the cytosol.[12] This contributes to the activation of a family of cysteine proteases called caspases, which are responsible for triggering the cellular morphological changes associated with apoptosis.[12]

The formation of the BAX/BAK pores is a critical regulatory step in the intrinsic apoptotic pathway. The change in protein conformation leading to BAX/BAK activation and the formation of mitochondrial pores can be triggered by a sub-class of pro-apoptotic proteins of the BCL-2 family called activator BCL-2 Homology Domain 3 (BH3)-only proteins (which include BH3-interacting domain death agonist and Bcl2-interacting mediator of cell death).[11,12] The formation of the BAX/BAK pore is also antagonized by their interaction with pro-survival BCL-2 proteins.[11,12] Because of their role as modulators of MOMP, the pro-survival proteins of the BCL-2 family are subjected to regulation by a variety of mechanisms.[13,14] For instance, sensitizer BH3-only proteins (Bcl-2-associated death promoter, NOXA and p53 upregulated modulator of apoptosis) bind and neutralize pro-survival BCL-2 proteins, preventing the latter from interfering with the formation of the BAX/BAK pores.[11–14] Pro-survival BCL-2 proteins are also subjected to post-translational regulation including phosphorylation,[13,14] deamidation[15] and limited proteolysis,[16] all of which have been shown to control the ability of these proteins to interfere with the initiation of apoptosis. Finally, another means of regulation of the pro-survival BCL-2 proteins is via the modulation of their turnover. This is the case of MCL-1, which is targeted by a system of ubiquitin-conjugating and deubiquitinating enzymes, resulting in the complex regulation of the half-life of this protein.[13,17]

The Sp2/0-Ag14 (Sp2/0) mouse hybridoma is acutely sensitive to a number of apoptosis-inducing stresses, including Gln starvation.[8,18] Remarkably, over 85% of Sp2/0 cells show apoptotic membrane blebbing and nuclear condensation within 2 h of Gln deprivation.[19] Moreover, cytochrome *c* leakage and BAX translocation to the mitochondria, two hallmarks of the intrinsic apoptotic pathway, are observed in Sp2/0 cells within 60 min of Gln deprivation,[19] suggesting that the anti-apoptotic BCL-2 family proteins expressed in this cell line are quickly inactivated upon Gln starvation. While our work has shown that Sp2/0 cells express low levels of BCL-2 and BCL-X_L,[8,20] whether other pro-survival BCL-2 family proteins are found in this cell line is unknown. Moreover, the contribution of these proteins to the survival of Sp2/0 cells, and their modulation following Gln deprivation, remain to be determined. In this paper, we show that MCL-1 is the principal pro-survival BCL-2 protein expressed in Sp2/0 cells, and that it plays an important role in determining the sensitivity of this cell line to Gln deprivation.

MATERIALS AND METHODS

Reagents and cell lines

Unless specified, all reagents were from Sigma Aldrich (Oakville, ON, Canada). The Sp2/0 mouse hybridoma and Ramos Burkitt lymphoma cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The BCL-X_L-overexpressing Sp2/0 cells and pTEJ8 vector-transfected control were described elsewhere.[8] The A431 epidermoid carcinoma and RIE-1 intestinal epithelial cell lines were kind gifts from Dr David Deperthes (MedDiscovery SA, Geneva, Switzerland) and Dr Kenneth D. Brown (Babraham Institute, Cambridge, UK), respectively. The Jurkat leukaemia cell line was obtained from Dr Leslie Sutherland (Health Sciences North, Sudbury, ON, Canada). The MCL-1 antibody was from Biolegend (San Diego, CA, USA). All other antibodies were from Cell Signaling Technology (Danvers, MA, USA). The BCL-2 protein inhibitors obatoclax and ABT-737 were purchased from Selleck Chemicals Inc. (Houston, TX, USA) and were prepared as 1000× stock solutions in dimethyl sulfoxide (DMSO).

Cell culture

A431 and RIE-1 cells were maintained in Dulbecco's modified Eagle's medium, while Ramos and Jurkat cells were cultured in Iscove's modified Dulbecco's medium (IMDM) and RPMI-1640 media, respectively. All media contained 4 mM Gln and were supplemented with 10% foetal bovine serum (Fisher Scientific, Whitby, ON, Canada) and 1% antibiotics (100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin). Sp2/0 cells were maintained in IMDM medium supplemented with 5% FetalClone I (FCS; Fisher Scientific), 1% antibiotics and 4 mM Gln. This medium will be referred to as complete IMDM. The BCL-X_L and pTEJ8-transfected Sp2/0 cells were cultured in complete IMDM containing 750µg ml^{-1} G-418 (Invivogen, San Diego, CA, USA).

For the glutamine deprivation experiments, exponentially growing Sp2/0 cells were washed twice in warm (37 °C) IMDM containing 5% FCS and 1% antibiotics, without Gln supplementation (hereafter referred to as Gln-free IMDM). The cells were then placed in Gln-free IMDM (4×10^5 cells ml^{-1}) and cultured for the indicated time periods. Controls were included in which Gln (4 mM) was added at the start of the experiment. For the determination of cell viability using Gln rescue assays, 1 ml aliquots were taken from each experimental group at the indicated time intervals. Gln was added (4 mM) to the amino acid-deprived aliquots, and culture was resumed for 24 h. Cell viability was then determined using the trypan blue dye exclusion assay.

The effect of obatoclax and ABT-737 on cell viability was determined by adding the drugs at the indicated concentration at the start of the experiment, and cell viability was determined 24 h later using the trypan blue dye exclusion assay. The control groups received DMSO instead of the inhibitors. To test the effect of obatoclax on the sensitivity of Sp2/0 cells to Gln starvation, the cells were cultured overnight in the presence of 25 nM obatoclax or an equivalent amount of DMSO.

The cells were then washed twice in warm Gln-free IMDM to remove the drug or DMSO and placed in Gln-free IMDM for 1 h. Control groups were supplemented with 4 mM Gln. After 1 h, Gln was added (4 mM) to the deprived cells. Culture was resumed for 24 h, and cell viability was determined using the trypan blue dye exclusion assay.

Western blot analysis

Total cell protein extracts were prepared in RIPA buffer [1% Igepal, 0·5% sodium deoxycholate, 0·1% sodium dodecyl sulfate, 0·2 mM sodium orthovanadate, 50 mM sodium fluoride, 0·1 mg ml^{-1} phenyl methyl sulfonyl fluoride and 1× Halt protease inhibitor cocktail (Fisher Scientific), in phosphate-buffered saline (9·1 mM Na_2HPO_4, 1·7 mM NaH_2PO_4, 150 mM NaCl and pH 7·4)], as described.[19] Proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto Immobilon PVDF membranes (Millipore, Billerica, MA, USA). Transfer efficiency was routinely monitored by staining the membranes with Ponceau S. For Western blot analysis, the membranes were blocked for 1 h at room temperature with Tris-buffered saline/Tween 20 (TTBS; 20 mM Tris base, 137 mM NaCl and 0·1% Tween 20, pH 7·6) containing 5% bovine serum albumin (BSA). The membranes were then exposed to the primary antibodies (diluted in 5% BSA/TTBS) for 1 h, washed three times in TTBS and exposed to a horseradish peroxidase-coupled secondary antibody for 1 h. After washing in TTBS, the membranes were processed for chemiluminescence detection using the Immobilon Western reagent (Millipore) and a Fluorchem 8000 imaging unit (Alpha Innotech, San Leandro, CA, USA).

Statistical analysis

Data were analysed by one-way analysis of variance and Scheffe's *post hoc* test.

RESULTS

Anti-apoptotic BCL-2-family protein expression in Sp2/0 cells

To better understand the modulation of apoptosis in Gln-starved Sp2/0 cells, we examined the expression levels of four pro-survival BCL-2 family proteins (BCL-2, BCL-X_L, BCL-W and MCL-1) in this cell line (Figure 1). Protein extracts from A431 and RIE-1 cells were also included for comparison purposes. Western blot analysis revealed low levels of BCL-2 in Sp2/0 cells and higher levels in RIE-1 cells. Similarly, while BCL-X_L could be seen in RIE-1 and A431 cells, much lower amounts were observed in Sp2/0 cells. BCL-W was observed in RIE-1 and A431 extracts but was found at very low levels in Sp2/0 cells. Interestingly, high levels of MCL-1 were present in Sp2/0 cells, while lower amounts were observed in RIE1 and A-431 protein extracts. Therefore, these data indicate that MCL-1 is the principal anti-apoptotic BCL-2 protein expressed in the Sp2/0 cell line.

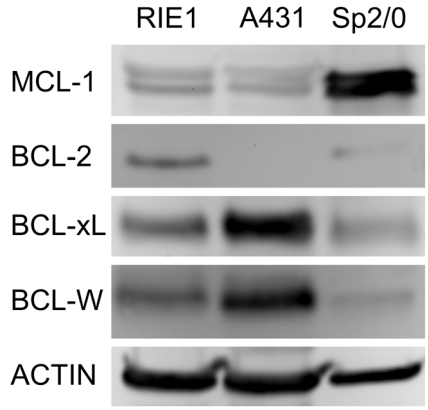

Figure 1. Myeloid cell leukaemia 1 (MCL-1) is the prominent pro-survival B-cell lymphoma 2 (BCL-2) protein expressed in Sp2/0-Ag14 (Sp2/0) cells. Protein extracts from A431, RIE-1 and Sp2/0 cells were processed for Western blot analysis and probed with antibodies against BCL-2, B-cell lymphoma extra-large, MCL-1 and BCL-2-like protein 2. β-actin was used as a protein loading control

MCL-1 levels are reduced in glutamine-deprived Sp2/0 cells

MCL-1 has a short half-life, the result of a complex regulation involving phosphorylation and ubiquitin-mediated proteolysis.[13,17] The presence of MCL-1 as the predominant pro-survival BCL-2 protein in Sp2/0 cells prompted us to examine its regulation following Gln deprivation. Several protein bands were detected with our MCL-1 antibody (Figure 2). Notably, three prominent protein bands of 36, 37 and 39 kDa, respectively, are of a similar size to those recently reported by Perciavalle *et al.*,[21] which they identified as different MCL-1 isoforms. The 37-kDa band likely corresponds to the commonly reported anti-apoptotic MCL-1L protein.[22] Two additional, minor bands were also seen on our immunoblots: a 26-kDa band, which is consistent with the expected size of the pro-apoptotic splicing variant MCL-1S,[22,23] and a 30-kDa band, which was shown by others to be a proteolytic product of the larger MCL-1L.[24] Therefore, several MCL-1 isoforms are found in Sp2/0 cells.

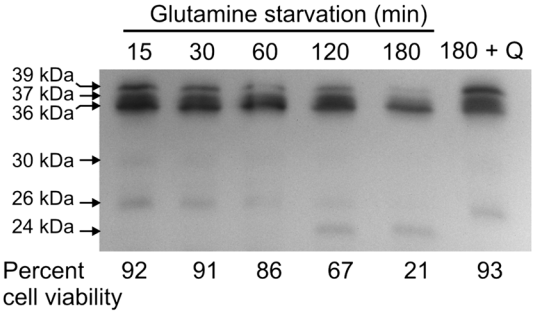

Figure 2. Modulation of myeloid cell leukaemia 1 (MCL-1) protein levels in L-glutamine (Gln)-starved Sp2/0-Ag14 cells. Sp2/0-Ag14 cells were cultured in Gln-free Iscove's modified Dulbecco's medium for the indicated time intervals. Cells cultured for 3 h in the presence of Gln (180 + Q) were used as control. Samples of each group were taken for the determination of cell survival using the Gln rescue assay. The remainder of the culture was processed for Western blot analysis of MCL-1 protein expression. Several putative MCL-1 isoforms are indicated by arrows

The effect of Gln starvation on MCL-1 protein levels in Sp2/0 cells was next examined in a time-course experiment. During the first 2 h of Gln starvation, the protein levels of all three major isoforms and the minor 26-kDa MCL-1 isoform did not significantly change (Figure 2). However, after 3 h of Gln deprivation, the levels of the 37- and 39-kDa MCL-1 isoforms had clearly decreased, while the 36-kDa isoform remained elevated. This was accompanied with the disappearance of the 26-kDa MCL-1 band and the appearance of a 24-kDa band (visible at the 2- and 3-h time points). The latter may correspond to a caspase-generated MCL-1 fragment.[25,26] Importantly, the decline in MCL-1 protein levels correlated with a significant decrease in Sp2/0 cell viability (Figure 2). Thus, these data show that the protein levels of several MCL-1 isoforms are reduced in Gln-deprived Sp2/0 cells in a time frame that coincides with the loss of cell viability.

MCL-1 inhibition by obatoclax triggers Sp2/0 cell death

To determine whether MCL-1 plays a role in the modulation of cell survival in Sp2/0 cells, we used two small molecule BH3 mimetics: ABT-737, which inhibits BCL-2, BCL-X_L and BCL-W but is ineffective against MCL-1,[27,28] and obatoclax, which has been shown to inhibit a broader spectrum of pro-survival BCL-2 family members, including MCL-1.[29,30] As controls, we used Jurkat and Ramos cells, two cell lines that have been shown by others to be sensitive to the effect of both BH3 mimetics.[31–33]

ABT-737 (15 μM) caused a significant reduction in Sp2/0 cell viability compared with the DMSO-treated cells (Figure 3A). ABT-737 was also toxic to both Jurkat and Ramos cells; however, a more pronounced effect was observed for the former, a phenomenon previously reported by others.[31] As expected,[32,33] both Jurkat and Ramos cells were sensitive to 15 μM obatoclax. Interestingly, the drug proved to be significantly more toxic to the Sp2/0 cell line, because no viable cells remained after treatment with 15 μM obatoclax.

To determine the minimal concentration of obatoclax, which is toxic to Sp2/0 cells, a dose–response experiment was carried out. Sp2/0 cells were cultured for 24 h in the presence of different concentrations of either drug, and cell viability was determined using the trypan blue assay. While ABT-737 was not toxic when used at concentrations of up to 1 μM, obatoclax caused a dose-dependent reduction in Sp2/0 cell viability, with concentrations of 250 nM causing over 90% cell death (Figure 3B). These results demonstrate that Sp2/0 cells are significantly more sensitive to the toxic effect of obatoclax than to ABT-737. They also indicate that MCL-1 plays an important role in the modulation of Sp2/0 cell viability.

Obatoclax sensitizes Sp2/0 cells to undergo cell death upon Gln starvation

We next sought to determine whether MCL-1 inhibition by obatoclax would sensitize Sp2/0 cells to Gln starvation. Sp2/0 cells were cultured for 24 h in the presence of a sub-lethal dose of obatoclax (25 nM). The cells were then washed and deprived of Gln for 1 h, which in itself is not sufficient to commit Sp2/0 cells to undergo apoptosis.[19] Cell viability was finally determined using a Gln rescue assay.

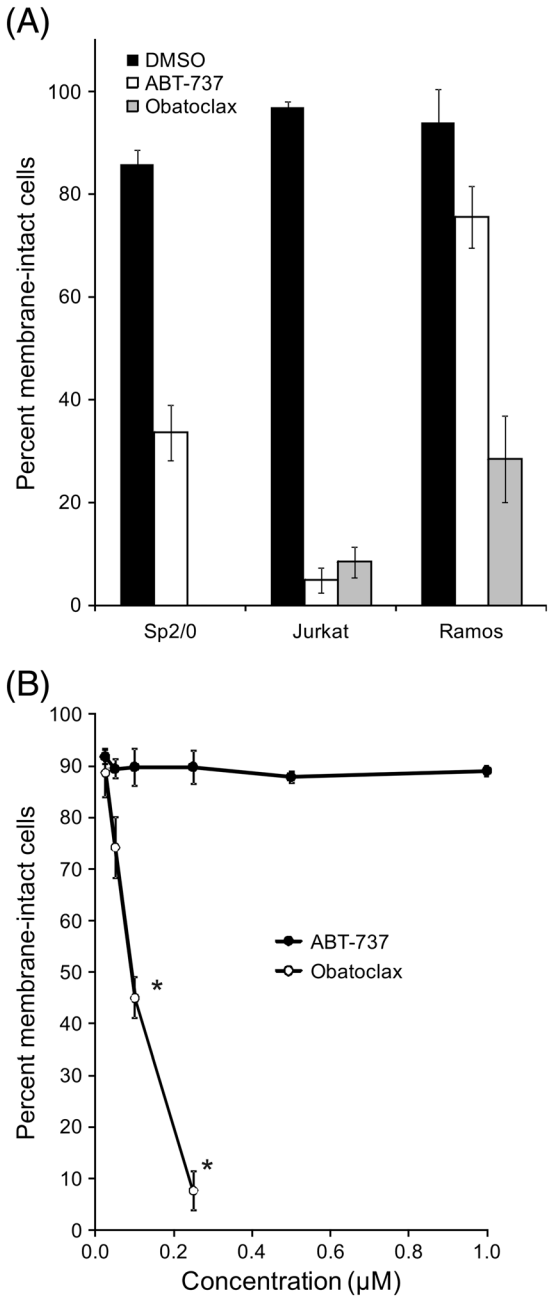

Figure 3. Effect of obatoclax and ABT-737 on Sp2/0-Ag14 (Sp2/0) cell survival. (A) Sp2/0, Jurkat and Ramos cells were cultured for 24 h in the presence of 15 μM of obatoclax or ABT-737. Cells cultured in the presence of an equivalent amount of dimethyl sulfoxide (DMSO) were used as control. Cell viability was determined using the trypan blue dye exclusion assay. (B) Sp2/0 cells were cultured for 24 h in the presence of the indicated amounts of obatoclax and ABT-737. Cell viability was then determined using the trypan blue assay. Data are the average ± standard deviation of three independent experiments. *$p < 0{\cdot}05$ versus sample treated with 0·025 μM obatoclax

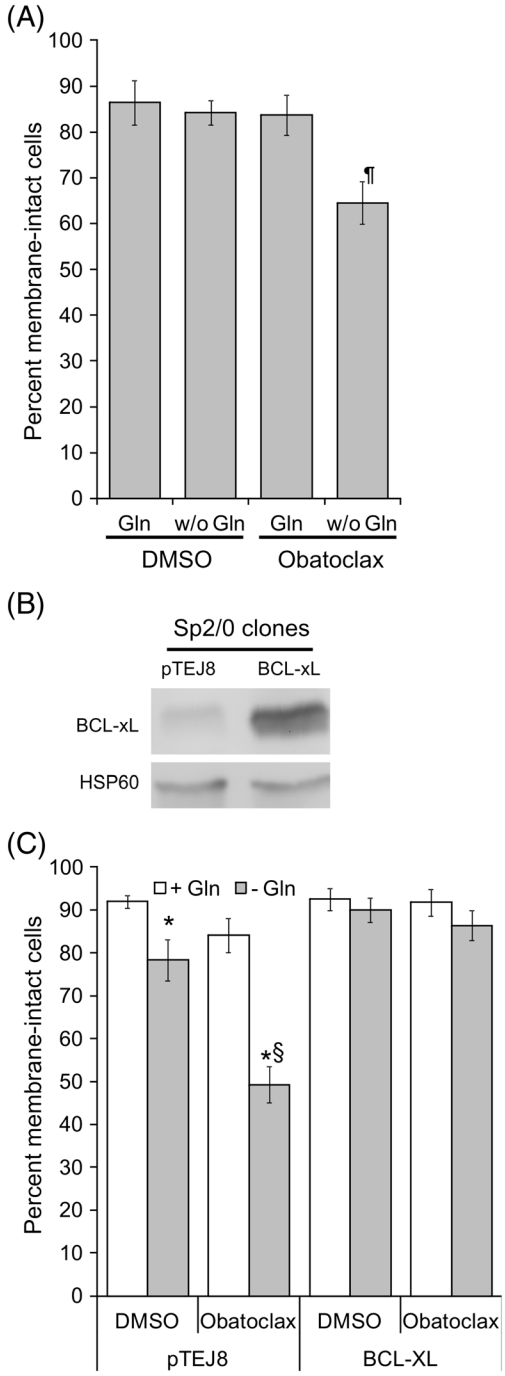

Figure 4. Obatoclax sensitizes Sp2/0-Ag14 (Sp2/0) cells to L-glutamine (Gln) starvation. (A) Sp2/0 cells were cultured for 24 h in complete Iscove's modified Dulbecco's medium, the presence of 25 nM obatoclax or an equivalent amount of dimethyl sulfoxide (DMSO). The cells were then washed and deprived of Gln for 1 h in the absence of obatoclax or DMSO. Control groups were supplemented with Gln at the start of the experiment. Gln was then added to the amino acid-deprived samples, and viability was assessed 24 h later by the trypan blue dye exclusion assay. ¶ $p < 0{\cdot}05$ versus Gln-supplemented control. (B) Expression of ectopic B-cell lymphoma extra-large (BCL-X_L) in transfected Sp2/0 cells and pTEJ8 vector control. HSP60 was used as a protein loading control. (C) BCL-X_L-overexpressing and vector-transfected Sp2/0 cells were processed as in (A). Data are the average ± standard deviation of three independent experiments. *$p < 0{\cdot}05$ versus corresponding Gln-supplemented control. §$p < 0{\cdot}05$ versus Gln-deprived/DMSO-treated control

As expected, the viability of cells treated with DMSO remained high, whether or not they were deprived of Gln (Figure 4A). However, a significant decrease in cell viability was observed in the Gln-deprived, obatoclax-treated cells.

In order to confirm that this increased sensitivity to Gln starvation by obatoclax was the result of the drug's BH3 domain-mimicking properties, Sp2/0 cells stably transfected with BCL-X_L or with the pTEJ8 vector were tested (Figure 4B). As observed with the wild type Sp2/0 cells, 25 nM obatoclax caused a significant increase in the sensitivity of the pTEJ8-control cells to a 1-h Gln starvation treatment (Figure 4C). Moreover, obatoclax did not sensitize BCL-X_L-overexpressing Sp2/0 cells to Gln deprivation, thus confirming that the drug's effect was through the inhibition of BCL-2 family proteins (Figure 4C).

DISCUSSION

Sp2/0 hybridoma cells show biochemical features of apoptosis within 30 min of Gln withdrawal, a process that is triggered by the intrinsic apoptotic pathway.[19] Because the pro-survival proteins of the BCL-2 family negatively regulate MOMP,[12] we sought to examine their involvement in the modulation of apoptosis in Gln-deprived Sp2/0 cells.

In this paper, we show that, of the four anti-apoptotic BCL-2 proteins surveyed, MCL-1 is by far the one with the highest level of expression in Sp2/0 cells (Figure 1). While this was not surprising, because MCL-1 expression has been demonstrated in a variety of haematopoietic cells,[13] this is, to our knowledge, the first report of expression of MCL-1 in a mouse hybridoma cell line.

MCL-1 has a short half-life,[13,17] which suggested a mechanism by which this anti-apoptotic BCL-2 protein could be rapidly inactivated in Gln-starved Sp2/0 cells. Our time-course experiments showed that Gln deprivation led to a decrease in the levels of the 37- and 39-kDa MCL-1 isoforms in a manner that coincided with the loss in cell viability, while the 36-kDa band remained high throughout the time course (Figure 2). In that regard, it is interesting to note that, while the two largest MCL-1 bands reside on the outer mitochondrial membrane and exhibit anti-apoptotic properties, the smaller, 36-kDa isoform localizes to the mitochondrial matrix, does not participate in the modulation of survival and is involved in the maintenance of the bioenergetic functions of the organelle.[21,34] Thus, Gln deprivation leads to the preferential degradation of the apoptosis-modulating MCL-1 variants in Sp2/0 cells.

The modulation of MCL-1 is complex and occurs at several levels, including transcriptional, post-transcriptional, translational and post-translational controls.[13] Our data indicate that in Sp2/0 cells, the inhibition of MCL-1 following Gln starvation occurs at least in part through a decrease in its protein levels. The appearance of a MCL-1-specific 24-kDa band (Figure 2) suggests that this may be achieved through the caspase-mediated cleavage of MCL-1.[25,26] However, the ubiquitylation and subsequent degradation of

MCL-1 by the proteasome, a well-characterized mode of regulation of this protein,[13,17] cannot be ruled out.

Our data indicate that the decrease in MCL-1 protein levels is most evident in Sp2/0 cells 2 h after Gln starvation (Figure 2). This coincides with other biochemical apoptotic events such as the degradation of the pro-survival protein X-linked inhibitor of apoptosis protein and caspase-3 activation.[19] However, the recruitment of BAX to mitochondria and the release of the mitochondrial apoptogenic proteins cytochrome *c* and SMAC/DIABLO into the cytosol occur as early as 1 h after Gln withdrawal.[19] Therefore, it is likely that the inhibition of MCL-1 in Sp2/0 cells involves other regulatory mechanisms. One possibility is that the function of MCL-1 is impeded through its interaction with modulatory proteins. For instance, the BH3-only proteins BIM and NOXA are known to bind to and block the anti-apoptotic function of MCL-1.[35,36] Another way whereby MCL-1 could be rapidly neutralized following Gln deprivation is through the modification of its phosphorylation status. Phosphorylation on Ser^{64}, triggered by the cyclin-dependent kinases CDK1 and CDK2 and by c-Jun N-terminal kinase, has been shown to be important to the anti-apoptotic activity of MCL-1.[37] In addition, phosphorylation of Ser^{162} is critical to the mitochondrial localization and anti-apoptotic function of MCL-1.[38] The recent demonstration of MCL-1 regulation by the protein kinase Mechanistic target of rapamycin,[39–41] the activity of which is controlled by glutaminolysis,[42] may provide a mechanistic link between intracellular glutamine levels and the cell survival machinery.

The correlation of the degradation of MCL-1 with the loss of Sp2/0 cell viability suggested that this BCL-2 family protein constitutes a significant barrier to the onset of cell death following Gln starvation. Using the BH3 mimetic obatoclax, we demonstrated that 250 nM of the drug was sufficient to kill Sp2/0 cells, while much larger amounts of ABT-737 (which does not target MCL-1) were required (Figure 3). Importantly, we found that as little of 25 nM obatoclax could significantly sensitize Sp2/0 cells to undergo cell death upon Gln starvation (Figure 4). Considering the current renewed interest in the use of inhibitors of glutaminolysis as anti-cancer agents,[43,44] our data raise the interesting possibility that the combined use of glutamine analogs and BH3 mimetics could improve treatment efficacy.[45]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1. Daye D, Wellen KE. Metabolic reprogramming in cancer: unraveling the role of glutamine in tumorigenesis. *Semin Cell Dev Biol* 2012; **23**: 362–369. doi:10.1016/j.semcdb.2012.02.002.
2. DeBerardinis RJ, Cheng T. Q's next: the diverse functions of glutamine in metabolism, cell biology and cancer. *Oncogene* 2010; **29**: 313–324. doi:10.1038/onc.2009.358.
3. DeBerardinis RJ, Mancuso A, Daikhin E, *et al.* Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. *Proc Natl Acad Sci U S A* 2007; **104**: 19345–19350. doi:10.1073/pnas.0709747104.
4. Wise DR, Thompson CB. Glutamine addiction: a new therapeutic target in cancer. *Trends Biochem Sci* 2010; **35**: 427–433. doi:10.1016/j.tibs.2010.05.003.
5. Eagle H, Oyama VI, Levy M, Horton CL, Fleischman R. The growth response of mammalian cells in tissue culture to L-glutamine and L-glutamic acid. *J Biol Chem* 1956; **218**: 607–616.
6. Mates JM, Segura JA, Martin-Rufian M, Campos-Sandoval JA, Alonso FJ, Marquez J. Glutaminase isoenzymes as key regulators in metabolic and oxidative stress against cancer. *Curr Mol Med* 2013; **13**: 514–534.
7. Fuchs BC, Bode BP. Stressing out over survival: glutamine as an apoptotic modulator. *J Surg Res* 2006; **131**: 26–40. doi:10.1016/j.jss.2005.07.013.
8. Charbonneau JR, Gauthier ER. Prolongation of murine hybridoma cell survival in stationary batch culture by Bcl-xL expression. *Cytotechnology* 2000; **34**: 131–139. doi:10.1023/A:1008186302600.
9. Ryder C, McColl K, Zhong F, Distelhorst CW. Acidosis promotes Bcl-2 family-mediated evasion of apoptosis: involvement of acid-sensing G protein-coupled receptor Gpr65 signaling to Mek/Erk. *J Biol Chem* 2012; **287**: 27863–27875. doi:10.1074/jbc.M112.384685.
10. Yuneva M, Zamboni N, Oefner P, Sachidanandam R, Lazebnik Y. Deficiency in glutamine but not glucose induces MYC-dependent apoptosis in human cells. *J Cell Biol* 2007; **178**: 93–105. doi:10.1083/jcb.200703099.
11. Czabotar PE, Lessene G, Strasser A, Adams JM. Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. *Nat Rev Mol Cell Biol* 2014; **15**: 49–63. doi:10.1038/nrm3722.
12. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol* 2008; **9**: 47–59. doi:10.1038/nrm2308.
13. Thomas LW, Lam C, Edwards SW. Mcl-1; the molecular regulation of protein function. *FEBS Lett* 2010; **584**: 2981–2989. doi:10.1016/j.febslet.2010.05.061.
14. Willimott S, Wagner SD. Post-transcriptional and post-translational regulation of Bcl2. *Biochem Soc Trans* 2010; **38**: 1571–1575. doi:10.1042/BST0381571.
15. Deverman BE, Cook BL, Manson SR, *et al.* Bcl-xL deamidation is a critical switch in the regulation of the response to DNA damage. *Cell* 2002; **111**: 51–62.
16. Kirsch DG, Doseff A, Chau BN, *et al.* Caspase-3-dependent cleavage of Bcl-2 promotes release of cytochrome *c*. *J Biol Chem* 1999; **274**: 21155–21161.
17. Mojsa B, Lassot I, Desagher S. Mcl-1 ubiquitination: unique regulation of an essential survival protein. *Cells* 2014; **3**: 418–437. doi:10.3390/cells3020418.
18. Perreault J, Lemieux R. Rapid apoptotic cell death of B-cell hybridomas in absence of gene expression. *J Cell Physiol* 1993; **156**: 286–293. doi:10.1002/jcp.1041560210.
19. Paquette JC, Guerin PJ, Gauthier ER. Rapid induction of the intrinsic apoptotic pathway by L-glutamine starvation. *J Cell Physiol* 2005; **202**: 912–921. doi:10.1002/jcp.20194.
20. Gauthier ER, Piche L, Lemieux G, Lemieux R. Role of bcl-X(L) in the control of apoptosis in murine myeloma cells. *Cancer Res* 1996; **56**: 1451–1456.
21. Perciavalle RM, Stewart DP, Koss B, *et al.* Anti-apoptotic MCL-1 localizes to the mitochondrial matrix and couples mitochondrial fusion to respiration. *Nat Cell Biol* 2012; **14**: 575–583. doi:10.1038/ncb2488.

22. Bingle CD, Craig RW, Swales BM, Singleton V, Zhou P, Whyte MK. Exon skipping in Mcl-1 results in a bcl-2 homology domain 3 only gene product that promotes cell death. *J Biol Chem* 2000; **275**: 22136–22146. doi:10.1074/jbc.M909572199.
23. Bae J, Leo CP, Hsu SY, Hsueh AJ. MCL-1S, a splicing variant of the antiapoptotic BCL-2 family member MCL-1, encodes a proapoptotic protein possessing only the BH3 domain. *J Biol Chem* 2000; **275**: 25255–25261. doi:10.1074/jbc.M909826199.
24. Soleymanlou N, Jurisicova A, Wu Y, *et al.* Hypoxic switch in mitochondrial myeloid cell leukemia factor-1/Mtd apoptotic rheostat contributes to human trophoblast cell death in preeclampsia. *Am J Pathol* 2007; **171**: 496–506. doi:10.2353/ajpath.2007.070094.
25. Herrant M, Jacquel A, Marchetti S, *et al.* Cleavage of Mcl-1 by caspases impaired its ability to counteract Bim-induced apoptosis. *Oncogene* 2004; **23**: 7863–7873. doi:10.1038/sj.onc.1208069.
26. Weng C, Li Y, Xu D, Shi Y, Tang H. Specific cleavage of Mcl-1 by caspase-3 in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in Jurkat leukemia T cells. *J Biol Chem* 2005; **280**: 10491–10500. doi:10.1074/jbc.M412819200.
27. Chauhan D, Velankar M, Brahmandam M, *et al.* A novel Bcl-2/Bcl-X(L)/Bcl-w inhibitor ABT-737 as therapy in multiple myeloma. *Oncogene* 2007; **26**: 2374–2380. doi:10.1038/sj.onc.1210028.
28. Oltersdorf T, Elmore SW, Shoemaker AR, *et al.* An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* 2005; **435**: 677–681. doi:10.1038/nature03579.
29. Nguyen M, Marcellus RC, Roulston A, *et al.* Small molecule obatoclax (GX15-070) antagonizes MCL-1 and overcomes MCL-1-mediated resistance to apoptosis. *Proc Natl Acad Sci U S A* 2007; **104**: 19512–19517. doi:10.1073/pnas.0709443104.
30. Zhai D, Jin C, Satterthwait AC, Reed JC. Comparison of chemical inhibitors of antiapoptotic Bcl-2-family proteins. *Cell Death Differ* 2006; **13**: 1419–1421. doi:10.1038/sj.cdd.4401937.
31. Meynet O, Beneteau M, Jacquin MA, *et al.* Glycolysis inhibition targets Mcl-1 to restore sensitivity of lymphoma cells to ABT-737-induced apoptosis. *Leukemia* 2012; **26**: 1145–1147. doi:10.1038/leu.2011.327.
32. Heidari N, Hicks MA, Harada H. GX15-070 (obatoclax) overcomes glucocorticoid resistance in acute lymphoblastic leukemia through induction of apoptosis and autophagy. *Cell Death Dis* 2010; **1**: e76. doi:10.1038/cddis.2010.53.
33. Martinez-Paniagua MA, Baritaki S, Huerta-Yepez S, *et al.* Mcl-1 and YY1 inhibition and induction of DR5 by the BH3-mimetic obatoclax (GX15-070) contribute in the sensitization of B-NHL cells to TRAIL apoptosis. *Cell Cycle* 2011; **10**: 2792–2805.
34. Huang CR, Yang-Yen HF. The fast-mobility isoform of mouse Mcl-1 is a mitochondrial matrix-localized protein with attenuated anti-apoptotic activity. *FEBS Lett* 2010; **584**: 3323–3330. doi:10.1016/j.febslet.2010.07.013.
35. Mahajan IM, Chen MD, Muro I, Robertson JD, Wright CW, Bratton SB. BH3-only protein BIM mediates heat shock-induced apoptosis. *PLoS One* 2014; **9**: e84388. doi:10.1371/journal.pone.0084388.
36. Pang X, Zhang J, Lopez H, *et al.* The carboxyl-terminal tail of Noxa protein regulates the stability of Noxa and Mcl-1. *J Biol Chem* 2014; **289**: 17802–17811. doi:10.1074/jbc.M114.548172.
37. Kobayashi S, Lee SH, Meng XW, *et al.* Serine 64 phosphorylation enhances the antiapoptotic function of Mcl-1. *J Biol Chem* 2007; **282**: 18407–18417. doi:10.1074/jbc.M610010200.
38. Thomas LW, Lam C, Clark RE, *et al.* Serine 162, an essential residue for the mitochondrial localization, stability and anti-apoptotic function of Mcl-1. *PLoS One* 2012; **7**: e45088. doi:10.1371/journal.pone.0045088.
39. Faber AC, Coffee EM, Costa C, *et al.* mTOR inhibition specifically sensitizes colorectal cancers with KRAS or BRAF mutations to BCL-2/BCL-XL inhibition by suppressing MCL-1. *Cancer Discov* 2014; **4**: 42–52. doi:10.1158/2159-8290.CD-13-0315.
40. Zang C, Eucker J, Liu H, Muller A, Possinger K, Scholz CW. Concurrent inhibition of PI3-kinase and mTOR induces cell death in diffuse large B cell lymphomas, a mechanism involving down regulation of Mcl-1. *Cancer Lett* 2013; **339**: 288–297. doi:10.1016/j.canlet.2012.11.013.
41. Koo J, Yue P, Deng X, Khuri FR, Sun SY. mTOR complex 2 stabilizes Mcl-1 protein by suppressing its GSK3-dependent and SCF-FBXW7-mediated degradation. *Mol Cell Biol* 2015. doi:10.1128/MCB.01525-14.
42. Duran RV, Oppliger W, Robitaille AM, *et al.* Glutaminolysis activates Rag-mTORC1 signaling. *Mol Cell* 2012; **47**: 349–358. doi:10.1016/j.molcel.2012.05.043.
43. Hensley CT, Wasti AT, DeBerardinis RJ. Glutamine and cancer: cell biology, physiology, and clinical opportunities. *J Clin Invest* 2013; **123**: 3678–3684. doi:10.1172/JCI69600.
44. Zhao Y, Butler EB, Tan M. Targeting cellular metabolism to improve cancer therapeutics. *Cell Death Dis* 2013; **4**: e532. doi:10.1038/cddis.2013.60.
45. Olsen RR, Mary-Sinclair MN, Yin Z, Freeman KW. Antagonizing Bcl-2 family members sensitizes neuroblastoma and Ewing's sarcoma to an inhibitor of glutamine metabolism. *PLoS One* 2015; **10**: e0116998. doi:10.1371/journal.pone.0116998.