Metabolic changes induced by DNA damage in Ramos cells: exploring the role of mTORC1 complex

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Double-strand breaks (DSB) are considered among the most cytotoxic damage on the DNA, inducing an essential set of molecular mechanisms to prevent and repair these breaks. The DSB induce the DNA damage response (DDR), which promotes cell cycle arrest, DNA repair mechanisms, and even induces apoptosis and cellular senescence [1]. Interestingly, this DNA repair machinery can be induced in different cells in an intentional and calculated way to generate genetic modification with diverse functionalities, such as neural development, and adult neurogenesis. Specifically, in B cells, the DDR induces B-cell differentiation [2,3].

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Abbreviations
4EBP1, eukaryotic translation initiation factor 4E-binding protein 1; ACSL3, acyl-CoA synthetase for long-chain fatty acids of family 3; ATM, ATM serine/threonine kinase; BCL2, B-cell lymphoma-2; DDR, DNA damage response; DSB, double-strand breaks; FAS, Fas cell surface death receptor; G6PD, glucose 6-phosphate dehydrogenase; GLUT1, glucose transporter 1; GLUT3, glucose transporter 3; HIF1, hypoxia 1 inducible factor; HK2, hexokinase 2; KI67, Antigen Ki-67; LDH, lactate dehydrogenase; MTA, mitochondrial transcription factor A; mTOR, mechanistic target of rapamycin; MIV, mevalonate kinase; p70S6K, ribosomal protein S6 kinase beta-1; PARP, poly (ADP-ribose) polymerase; PCNA, proliferating cell nuclear antigen; PDH, pyruvate dehydrogenase; PDK1, pyruvate dehydrogenase kinase 1; PFKP, phosphofructokinase; PGD, phosphogluconate dehydrogenase; PPP, Pentose phosphate pathway; RPE, ribulose phosphate 3-epimerase; RPIA, ribose 5-phosphate isomerase A; SC5D, sterol-C5-desaturase; SCD1, stearoyl-CoA desaturase.
where the transcriptional repressor BCL6 regulates this process avoiding cellular transformation, but any genetic or epigenetic alteration may induce cancer, such as B-cell lymphomas.

DNA damage is associated with metabolic changes, increasing the nucleotide synthesis and anabolic glucose metabolism, and reducing glutamine anaplerosis [6,7]. However, there is little information about the signaling pathway induced by DSBs and the metabolic changes triggered by these breaks in B cells.

One of the most critical metabolic regulators in many cells is the mechanistic target of rapamycin (mTOR), which is a serine/threonine kinase activated by growth factors, nutrients, and amino acids, which controls fundamental cellular processes such as cell growth, protein synthesis, aging, autophagy, survival, and metabolism [6,7]. mTOR forms two distinct multiprotein complexes; mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), containing mTOR, Raptor, mLST8 (GβL), PRAS40, and Depotor; and mTOR, Rictor, Sin1, mLST8, Depotor, and PROTOR, respectively [8,9]. The activation of mTORC1 induces the phosphorylation of two principal translational regulators, S6K and 4EBP1, to increase ribosome biogenesis and protein synthesis. The activation of mTORC2 regulates the actin cytoskeleton, proliferation, and survival. mTORC1 is sensitive to acute rapamycin-induced inhibition, while mTORC2 is insensitive to this treatment, but several evidences suggest that a prolonged rapamycin treatment could inhibit mTORC2 in several cell lines [10,11].

mTORC1 signaling has been associated with nucleotide synthesis, specifically promoting de novo pyrimidine and purine synthesis through several mechanisms [12,13], suggesting that this pathway is also crucial in the regulation of another significant metabolic pathway, the pentose phosphate pathway (PPP). mTORC1 also appears to play a crucial role in lipid synthesis, inducing the activation of lipogenic gene expression and increasing lipid synthesis [14,15]. Besides, the mTORC1 pathway induces glycolysis and glutaminolysis processes to support anabolic functions, inducing the expression of glucose transporters and many other enzymes associated with these pathways as well [16].

In the immune system, mTORC1 has been connected to T- and B-cell differentiation, migration, tolerance, and antibody production [17–20]. In B cells, the activation of mTORC1 results in an increase in glucose uptake and ribosomal biogenesis [21]. Indeed, this pathway is activated in positively selected B cells, inducing an increase in anabolic pathways; in the same way, decreases in mTORC1 activity are related to cessation of proliferation [22]. Even when, in B cell, mTORC1 signaling is critical for establishing an effective Ab production, there is no evidence whether DNA damage associated with these events is related to mTORC1 signaling.

The principal aim of this work was to evaluate the metabolic changes induced by etoposide, which induces DSB in Ramos cells. We determined whether DSB can regulate mTORC1 signaling. Also, we analyzed the effect of the direct inhibition of the mTORC1 pathway on metabolism pathways using rapamycin. Interestingly, the effect of DSB and rapamycin on metabolic gene expression was similar in several genes, suggesting that mTORC1 inhibition could be involved in regulating these genes when DSBs occur. Additionally, we analyzed the effect of DSB and the inhibition of mTORC1 on survival processes. DSB and rapamycin present significant differences in the modulation the expression of several genes associated with cell apoptosis and cell proliferation, suggesting that DSB requires different signaling pathways to promote these events. These results provided important information about DSB molecular mechanism in association with mTOR pathway.

Materials and methods

Cell culture

Ramos cells are a B lymphocyte cell line from Burkitt’s Lymphoma (ATCC CRL-1596™). This cell line was grown in RPMI-1640 (from Corning) supplemented with 10% fetal bovine serum (from Biowest, FL, USA), 100 U·mL⁻¹ penicillin, 100 mg·mL⁻¹ streptomycin, and 2 μM l-glutamine (from Hyclone, Logan, UT, USA), 1 mM sodium pyruvate, and essential amino acids (from Corning, Glendale, AZ, USA), at 37 °C in humidified 5% CO₂ atmosphere.

Western blot analysis

Cells were cultured and treated with 20 μM etoposide (from Sigma, Darmstadt, Germany) or 50 nM rapamycin (Calbiochem, San Diego, CA, USA) for different periods. Then, cells were lysed in lysis buffer (50 mM Tris–HCl pH 7.4; 150 mM NaCl; 1 mM EDTA; 1% Triton X-100, 1 mM Sodium orthovanadate, 100 μg·mL⁻¹ PMSF, 2 μg·mL⁻¹ aprotinin, 2 μM leupeptin, and 1 μg·mL⁻¹ pepstatin), and protein concentration was determined using the Bradford assay. Protein extracts were resolved by SDS/PAGE, 100 or 60 μg per lane, on a 6% or 12% polyacrylamide gels and transferred into Nitrocellulose Membrane, 0.45 μm (Bio-Rad Laboratories, CA, USA). Next, the membrane was blocked with 5% bovine albumin serum or 5% skimmed milk, and then membranes were incubated with 1 : 5000 dilutions of primary antibodies against p-4EBP1, p-P70S6K, p-mTOR, or total proteins to evaluate the
mTOR pathway (Cell Signaling Technology, Inc., Danvers, MA, USA; cat No.2855; cat No.9234; cat No.5536, respectively). Cleaved caspase 3 and cleaved PARP (Cell Signaling Technology, Inc.; cat No.9661, cat No. 5625, respectively) to determine apoptosis marker. Antibodies against p-ATM and total ATM to evaluate DNA damage (Cell Signaling Technology, Inc.; cat No.4526 and cat No.2873, respectively). Tubulin (Calbiochem, Darmstadt, Germany, cat No. CP06) was used as loading control.

Quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR)

Total RNA was isolated from cells using Trizol reagent (Life Technologies, Waltham, MA, USA) following the manufacturer’s instructions. Total RNA was subjected to RT-PCR. 1–5 µg of total RNA was used to synthesize first-strand cDNAs with the iScript kit (Bio-Rad, CA, USA). Quantitative RT-PCR (RT-qPCR) analysis was performed as described previously [23]. Expression was normalized to 36B4 mRNA expression as a housekeeping gene. Oligonucleotide primers for real-time RT-qPCR are described in Table A1 in the Appendix section [24].

Glucose transport assay

Transport assay was performed using a glucose analog, 2-deoxy-ß-D-Glucose (2-DOG), to measure glucose uptake in treated Ramos cells. The assay was carried out in a final volume of 200 µL, where 30 µL corresponded to a suspension of cells previously treated for different periods of time with etoposide or rapamycin, or control cells using only DMSO, the vehicle. The cell suspension corresponds to approximately 10 million of cells in PBS at pH 7.4, supplemented with 1 mM CaCl2 and 0.5 mM MgCl2. The remainder (170 µL) corresponds to a radioactive mixture containing 0.5 µCi of 2-DOG-[3H], 0.25 mM 2-DOG at cold and 1× PBS pH 7.4 supplemented 1 mM CaCl2 and 0.5 mM MgCl2 at 4 °C. To perform the test, 30 µL of cells with 170 L of radioactive mixture was mixed, and after 30 s the reaction was stopped by adding 1 mL of stop solution (PBS 1× pH 7.4 supplemented with 1 mM CaCl2 and 0.5 mM MgCl2 at 4 °C). For the basal uptake controls at time 0, the stop solution was added to the cells before the radioactive mixture. Cells were collected by centrifugation at maximum speed for 10 s, the supernatant was removed, and the cell pellet was washed with 1 mL of the stop solution, then the cells were centrifuged again under the same conditions, and the supernatant was removed. The resulting pellets were suspended in 200 µL of cell lysis buffer (50 mM Tris–HCl pH 7.4; 150 mM NaCl; 1 mM EDTA; 1% Triton X-100) and left overnight. The next day the lysate was transferred to a vial, and 2 mL of scintillation fluid was added to the tube. Finally, the radioactivity was measured using a PerkinElmer Tri-Carb 2910 TR counter (OH, USA). To determine whether the glucose uptake by Ramos cells was dependent on the GLUTs transporters, we analyze the effect of cytochalasin B. Briefly, cytochalasin B was added to the radioactive mixture at a final concentration of 1 µM.

Cell viability assay using propidium iodide

Cells were cultured in a 96-well plate in 100 µL of RPMI-1640 without phenol red (from Corning), supplemented with 10% fetal bovine serum (from Biowest), 100 U·mL−1 penicillin, 100 mg·mL−1 streptomycin and 2 mM L-glutamine, (from Hyclone), 1 mM Sodium Pyruvate, and essential amino acids (from Corning), at 37 °C in humidified 5% CO2 atmosphere. After 24 h, the cells were treated with different concentrations of etoposide or rapamycin. N,N-dimethylformamide was used as a positive death control. Once the treatment times were over, it was added 100 µL of HBSS modified-Ca2+ (40 µM KH2PO4; 30 µM NaH2PO4, PO4·H2O; 13.6 µM NaCl; 600 µM L-glucose; 500 µM KCl; 900 µM CaCl2) with propidium iodide (from Thermo Fisher Scientific, Eugene, OR, USA) to a final concentration of 5 µM per well. On the contrary, in control death wells, 2 µL from a 500 µM propidium iodide stock was added. The plates were incubated at 37 °C for 5 min, and the plate was measured on a fluorescent plate reader using an excitation wavelength of 530 nm and an emission wavelength of 620 nm.

Statistical analysis

Data are presented as mean ± SD in the case of qPCR, western blot, and viability plots, and as mean ± SE and in the case of radioactive transport plots of the values from the number of experiments performed in triplicate. Data were analyzed for statistically significant differences using the Mann–Whitney test (Student’s t-test) or ANOVA (Tukey’s test) as indicated in the corresponding figures. The data were graphed and analyzed using the GRAPHPAD PRISM 6 program (San Diego, CA, USA).

Results

DNA damage modulates the expression of metabolic genes in Ramos cells

To evaluate the effect of DSB on gene transcripts level of the different metabolic pathways, we used etoposide and RT-qPCR assays to analyze the expression of different genes that actively participate in glucose metabolism, pentose phosphate pathway, lipid and cholesterol synthesis, and some mitochondrial genes [24]. First, to test DNA damage induced by etoposide, we treated Ramos cells with this compound during different times, and we analyzed the phosphorylation of an important DNA damage sensor such as ATM and the increase in γH2AX.
In Fig. 1A,B, we can see that 20 \( \mu \)M of etoposide can induce a clear increase in ATM phosphorylation levels at 3 h, suggesting that this drug can induce DNA damage in this model; also there is a significant increase in the levels of \( \gamma \)-H2AX at the same time point. These markers decrease after 6 h treatment. Then, the Ramos cells were treated with etoposide for 3 h; etoposide induces significant changes in the expression of several genes associated with proliferation at this time [2]. In respect to glucose metabolism genes (Fig. 1C), we evaluated the expression of glucose transporter 1 (GLUT1), glucose transporter 3 (GLUT3), pyruvate dehydrogenase kinase 1 (PDK1), and phosphofructokinase (PFKP). A significant decrease in the transcript levels was seen after inducing DNA damage in all these genes, highlighting that the most affected genes were GLUT1 and GLUT3, which decreased at least 50%. As a control, we analyzed the expression of TCL1 gene because it has been previously demonstrated that etoposide decreases the expression of this gene at 20 \( \mu \)M for 3 h of treatment in Ramos cells.

A similar effect was observed in the expression of genes associated with pentose phosphate pathways...
(Fig. 1D), where the expression of glucose 6-phosphate dehydrogenase (G6PD), ribulose phosphate 3-epimerase (RPE), ribose 5-phosphate isomerase A (RPIA), and phosphogluconate dehydrogenase (PGD) genes were evaluated. In this case, the treatment with etoposide induced a significant decrease in at least 20% in the transcript levels of the RPE, RPIA, and PGD genes, while the expression of the G6PD gene had no variation with respect to the control cells.

To analyze the genes related to the synthesis of lipids and cholesterol (Fig. 1E), the expression of acyl-CoA synthetase for long-chain fatty acids of family 3 (ACSL3), mevalonate kinase (MVK), stearoyl-CoA desaturase (SCD1), and sterol-C5-desaturase (SC5D) genes were evaluated. In this case, the treatment with etoposide was differentially modulating the expression of these genes. To ACSL3 and SCD1 gene expression, we saw a significant decrease in at least 20% in their transcript levels, while the MVK gene expression did not show variations with respect to the control. Interestingly, the expression of the SC5D gene increased significantly by approximately 70% with the treatment.

Regarding the mitochondrial genes (Fig. 1F), mitochondrial transcription factor A (MTAF), hypoxia 1 inducible factor (HIF1), hexokinase 2 (HK2), lactate dehydrogenase (LDH), and pyruvate dehydrogenase (PDH) were evaluated. There was a significant decrease of at least 10% in the transcript levels of the HIF1, HK2, LDH, and PDH genes, while there were no significant variations in MTAF gene expression.

Overall, our results demonstrate that etoposide can modulate the expression of various genes associated with different metabolic pathways in Ramos cells.

**Etoposide inhibits mTOR signaling pathway**

Since mTOR signaling has a vital role in metabolic changes, we evaluate the effect of DSB, induced by etoposide, on the phosphorylation state of mTOR and some classical targets of the mTOR signaling pathway in Ramos cells. For this event, we measured the level of mTOR phosphorylated on Ser 2448, a key site for kinase activity [25], and the phosphorylation levels of two principal mTORC1 targets: (a) P70S6K in its residue Thr389, which is the key of its kinase activity [26] and (b) 4EBP1 in its Thr37/46 residues, which should be noted, do not prevent the binding of 4EBP1 to eIF4E, but allow subsequent phosphorylation of other sites which are associated with the initiation of translation process [27]. As shown in Fig. 2A,B, our results demonstrated that the treatment with etoposide induces a decrease in mTOR phosphorylation levels in a time-dependent way. Analyzing the specific target of mTOR; P70S6K, the treatment with etoposide also induces a decrease in P70S6K phosphorylation levels. Concerning 4EBP1, in this case, the etoposide has a low effect on 4EBP1 phosphorylation levels, inducing a significant decrease only at 3 h post-treatment. These results suggest that etoposide induces a decrease in phosphorylation degree on mTORC1 targets, suggesting that this drug can decrease the activity of this pathway in Ramos Cells.

**Rapamycin modulates the expression of metabolic genes in Ramos cells**

Since that etoposide induced a clear decrease in mTORC1 signaling, we proceeded to evaluate the expression of the same pool of metabolic genes by RT-qPCR, but using cells treated with 50 nm of rapamycin, an inhibitor of mTOR. First, to analyze the effect of rapamycin in these cells, we study mTOR signaling pathway under rapamycin treatment. As shown in Fig. 2C, our results demonstrated that the treatment with rapamycin induces a decrease in phosphorylation levels of two classical targets of mTORC1. The treatment with rapamycin induces a clear decrease in P70S6K phosphorylation levels. Concerning 4EBP1, in this case, the rapamycin induced a decrease in 4EBP1 phosphorylation after 3-h treatment. These results demonstrate that rapamycin induces a clear inhibition of mTORC1 signaling in Ramos Cells.

With respect to the genes associated with glucose metabolism (Fig. 2D), the behavior is similar to that observed with etoposide (Fig. 1C), with a significant decrease of at least 20% in transcript levels of TGL1 and a decrease at least 40% of all glycolytic genes evaluated, except for the GLUT3 gene, that in this case does not show variations in its expression with respect to the control.

Regarding the genes of the pentose phosphate pathway (Fig. 2E), there was no variation in the expression of the G6PD gene with respect to the control. A significant decrease in the RPE, RPIA, and PGD genes was seen; however, the decrease in these genes’ transcript levels was more significant with rapamycin than etoposide.

In the case of genes of lipid and cholesterol synthesis (Fig. 2F), the behavior was different from that observed with etoposide (Fig. 1E) in most of the genes analyzed. Although the transcript levels of the ACSL3 and SCD1 genes showed the same tendency as in the previous case, the decrease in the SCD1 gene expression was more significant with rapamycin by approximately 50% than with etoposide. On the contrary, a significant decrease of approximately 40% in the MVK gene expression was observed with rapamycin, but not...
with etoposide. The most surprising change was in the transcript levels of the \textit{SC5D} gene, which increase its expression with etoposide, but showed a clear decrease of approximately 30% with rapamycin.

In the case of genes associated with mitochondrial function (Fig. 2G), the treatment with rapamycin also induced similar results than etoposide, with some exceptions (Fig. 1F). In this case, the treatment with rapamycin induces a significant decrease of approximately 20% in \textit{MTAF} gene expression. Concerning \textit{HIF1} and \textit{HK2} genes, rapamycin treatment induces a remarkable decrease in gene expression, especially for the \textit{HK2} gene, where the expression decreased approximately 70%. Regarding the \textit{LDH} and \textit{PGD} genes, the expression of both genes decreased in the presence of rapamycin.

Overall, our results suggest that rapamycin treatment showed similar behavior to etoposide treatment on the gene expression of several genes analyzed.

**Etoposide and rapamycin affect glucose uptake, mediated by GLUTs**

Although a similar effect has been observed in the treatments of etoposide and rapamycin on the expression of different genes of the metabolic pathways, it is essential to know whether these drugs have a similar effect at the functional level, specifically glucose transport, which is necessary for the proliferation and differentiation processes, principally due to the decrease in \textit{GLUT1} transcript levels observed previously (Figs 1 and 2).
The glucose uptake was measured in Ramos cells using a non-metabolizable glucose analog, 2-DOG, radioactively labeled. The uptake was measured at different periods. These kinetic analyses show that the glucose incorporation rate was linear until 30 s (Fig. 3A). The next step was to assess whether the transport of glucose from Ramos cells was mediated primarily by facilitative glucose transporters, GLUTs (Fig. 3B). For this aim, we treated Ramos cells only with the vehicle, DMSO, or with 20 μM etoposide for 3 h, and we used cytochalasin B (CCB), a well-known inhibitor of GLUTs transporters. As we can see, the treatment with etoposide does not change the glucose analog transport at 3 h with respect to the control. However, in both cases, control cells or treated with etoposide, the incubation with CCB induce a significant decrease, in approximately 80% and 70%, respectively, in the uptake of 2-DOG. These results indicate the high dependence of this process on the GLUTs transporters in Ramos cells.

Subsequently, to evaluate the effect of etoposide or rapamycin on glucose uptake in Ramos cells, we...
treated the cells with 20 µM etoposide or 50 nM rapamycin for different periods. The effect was evaluated both early (Fig. 3C) and late (Fig. 3D) times of treatment. Our results show a clear difference in the effect of both drugs. Regarding the early times, as can be seen in treatments of 1 and 6 h (Fig. 3C) and 3 h (Fig. 3B) of etoposide does not affect glucose analog uptake. In contrast, 6 h of treatment with rapamycin induced a significant decrease in glucose transport of these cells of approximately 80%. However, the treatment at late times (24 h) with this drug; the uptake is restored (Fig. 3D). On the contrary, in late treatment times, etoposide showed a significant decrease in glucose transport, approximately 50%. Our results showed that glucose transporters GLUTs mainly mediate the glucose uptake in Ramos cells, and etoposide and rapamycin affect this metabolite’s uptake in Ramos cells differently; rapamycin inhibits the uptake at early times of treatment, and etoposide inhibits the glucose uptake at late times.

The effect of etoposide and rapamycin on the regulation of apoptosis and proliferation genes

Since the decrease in metabolic rate, especially glucose uptake, is associated with cell death activation, we evaluated the effect of etoposide and rapamycin on the gene expression of several genes associated with apoptosis (Fig. 4A,B) and proliferation processes (Fig. 4C,D). To study the apoptosis cell death, we evaluated the expression of FAS, NOXA, PUMA, BCL2, and BAX genes. We observed that both etoposide and rapamycin affect the expression of these genes but in a different way. Etoposide triggers a significant increase in the expression of FAS and NOXA genes (Fig. 4A). In the expression of FAS, there was an increase in transcription levels of approximately eleven times greater than the control at 3 h. For NOXA, there was an increase of five times at 6 h. On the contrary, rapamycin treatment showed a clear increase of approximately five times in the PUMA gene expression.

Fig. 4. DNA damage and mTORC1 inhibition affect the expression of genes associated with apoptosis and cell proliferation. Ramos cell line was treated with 50 nM rapamycin or 20 µM etoposide for different periods of times. (A) Effect of etoposide on apoptotic gene expression. (B) Effect of rapamycin on apoptotic gene expression. (C) Effect of etoposide on proliferation gene expression. (D) Effect of rapamycin on proliferation gene expression. 36B4 was used as a housekeeping gene and DMSO was used as vehicle. The results are representative of three independent experiments. Error bars indicate SD, P-values determined by Mann–Whitney test (nonparametric t-test). *P < 0.05.
at 6 h (Fig. 4B). Regarding the BCL2 gene, there are no differences with respect to the control with any treatments, and the results related to the BAX gene show a significant decrease in both treatments at all the times analyzed (Fig. 4A,B).

Then, Ki67, MCM2, and PCNA proliferation genes were analyzed; in this case, it was detected that both treatments affect the expression of these genes in a very similar manner (Fig. 4C,D), showing that there was no variation in the levels of transcription for the MCM2 gene with respect to the control, but we observed a significant decrease in the transcripts of Ki67 and PCNA with both drugs.

Thus, etoposide and rapamycin modulate apoptosis genes differently, each having specific targets but regulating proliferation genes in a similar inhibitory manner.

**The effect of etoposide and rapamycin on cell viability**

Etoposide and rapamycin induce evident variations in the expression of different genes associated with the apoptosis process, suggesting that these stimuli induced cell death. To test this hypothesis, viability assays were carried out using propidium iodide at 24, 48, and 72 h of treatment. As we can see in Fig. 5A, the treatment with etoposide significantly affects Ramos cells viability, increasing cell death by approximately 10% with concentrations of 20 µM at 48 h of treatment and 30%, with concentrations of 100 µM of this drug at 72 h of treatment. The results show that cell death is time and concentration dependent. Using different concentrations of rapamycin, we could see that this drug also can induce an increase in cell death, which was significant using 300 nm at 48-h treatment, and 50 nm or more at 72-h treatment (Fig. 5B).

Finally, we determined the levels of protein markers associated with apoptosis using etoposide or rapamycin at 3, 6, and 24 h post-treatment; specifically, evaluating the levels of cleaved caspase 3 and cleaved PARP with both treatments. The treatment with etoposide induces a significant increase in the levels of cleaved caspase 3 at 6 h post-treatment. Similar results were obtained on the levels of cleaved PARP with a clear increase at 3 h post-treatment (Fig. 5C). The

![Fig. 5. Effect of etoposide and rapamycin on cell viability. Ramos cell line was treated with different concentrations of etoposide or rapamycin at different time-periods. (A) Effect of etoposide on the cell viability after 24, 48, and 72 h of treatment. DMSO was used as negative control and DMF (25% Dimethylformamide and 25% SDS) as positive death control. (B) Effect of rapamycin on the cell viability after 24, 48, and 72 h of treatment. DMSO was used as negative control and DMF (25% Dimethylformamide and 25% SDS) as positive death control. (C) Effect of etoposide 20 µM on cleaved Caspase 3 and cleaved PARP levels. (D) Effect of rapamycin 50 nM on cleaved Caspase 3 and cleaved PARP levels. These results are representative of three independent experiments. The results are representative of three independent experiments. Data are presented as mean ± SE, P-values determined by ANOVA. *P < 0.05.](image-url)
treatment with rapamycin also induces an increase in cleaved caspase3 and fragmented PARP (Fig. 5D). These results demonstrate that the increase in cell death using etoposide and rapamycin is accompanied by an increase in classical apoptotic markers.

Discussion

DNA damage induces the activation of several signaling pathways, most of them associated with repairing and cell death; however, this event may induce many other cellular responses. In the current study, we evaluate the metabolic changes during DSB in Ramos cells, analyzing the gene expression profile of enzymes associated with different metabolic pathways. Also, in this work, we evaluated the regulation of mTORC1 pathway induced by etoposide and its possible role in the metabolic changes observed.

Cancer cells or highly proliferative cell lines take up more substantial amounts of glucose than normal cells and metabolize it by glycolytic pathway at high rates [28]. In this work, we used the Ramos cell line, a B lymphocyte from Germinal Center (GC). GC is a site of high rate of proliferation associated with the increase in glucose uptake and ribosomal biogenesis [21]. It has been demonstrated that DNA damage, induced by DNA rearrangement, promotes B-cell differentiation into long-lived plasma cells and memory B cells [2], as we have known the differentiation process involves downregulation of glycolysis pathway and upregulation of oxidative phosphorylation (oxphos) pathway [29].

We found that DSB induced by etoposide in Ramos cells decreased the expression of several metabolic enzymes associated with glucose metabolism, the pentose phosphate pathway, and some genes associated with mitochondrial function and lipid and cholesterol synthesis. Also, in these cells DNA damage induces similar results in other cell models; specifically, DNA damage induces the activation of several DNA damage sensors like ATM that induces the activation of p53 by phosphorylation [30], which has been directly associated with GLUT1 and GLUT4 gene repression. Interestingly, we demonstrated that DSB induces a clear decrease in glucose uptake in this cell model, which is related to the decrease in GLUT1 and GLUT3 expression induced by etoposide. Moreover, Poly (ADP-ribose) polymerase 1 (PARP1), which also acts as a DNA damage sensor and is quickly activated in response to DNA damage [31,32], has been related to affect metabolism; PARP has also been reported to inhibit hexokinase (HK), a critical enzyme in the glycolytic pathway [33,34]. It has been demonstrated that PARP activation shifts the metabolic reliance to oxphos, which has been suggested that it is critical for damaged cell survival [31]. Also, it is well known that metabolism impacts DDR pathway via the regulation of metabolite pools; both glycolysis and glutaminolysis promote DSB repair [35]. Our results also have shown a decrease in the expression of several genes associated with the pentose phosphate metabolic pathway, which is crucial for nucleotide synthesis and DNA repair. We could see a decrease specifically for RPE, RPIA, and PGD, but not in G6PD. Interestingly, it has been demonstrated that the activation of p53 can inhibit the G6pd activity, which is a limiting enzyme of this pathway [36]. Therefore, it will be essential to determine the effect of DNA damage on the G6pd activity in future. Also, our results provide evidence that DNA damage induces a downregulation in the expression of ACSL3 and SCD1, both involved in fatty acid metabolism, which is often dysregulated in highly proliferative and cancer cells [37,38], unlike what happens with the case of the MVK and SCD5 enzymes that have not been related to cancer prognosis [39].

There is plenty of evidence demonstrating that mTOR signaling can modulate several metabolic pathways, including glycolysis, pentose phosphate, and lipid biosynthesis [15]. The effect of DNA damage on mTOR signaling in other models has been studied with controversial results [40–43]. It has been demonstrated that mTOR is a signaling pathway involved in DNA repair. Indeed, there is evidence that the inactivation of mTOR induces the autophagy process and promotes the transcriptional expression of damage-regulated autophagy modulator (DRAM), which is a lysosomal protein that facilitates the autophagy. Depending on the extent of DNA damage, autophagy plays a cytoprotective or cytotoxic role in cancer.

In this work, we demonstrated that damage induced by etoposide triggers a downregulation of mTOR signaling, inducing a decrease in phosphorylation on mTOR and its classical target; P70S6K in Ramos cells. mTOR is frequently deregulated in human cancer, and indeed mTOR inhibitors have been commonly used to treat human malignancies [21]. It is estimated that between 60% and 80% of all cancers have active mTORC1 signaling. The mTOR is a serine-threonine kinase critically involved in controlling anabolism and metabolic reprogramming in immune cells [44]. Different stimuli, including glucose, growth factors, and other signals, can activate mTOR complex 1 (mTORC1), which induce cell growth [45]. To analyze the effect of mTORC1 signaling, we use rapamycin as an mTOR inhibitor. As we expect, the effect of rapamycin on the metabolic pathways was very similar to
that induced by DNA damage, suggesting that down-regulation of mTORC1 is involved for this metabolic switch. However, we demonstrate that etoposide affects GLUT3 gene expression, but rapamycin does not, suggesting that etoposide and rapamycin probably have a different or parallel mechanism to exert their inhibitory effect on glucose uptake in these cells. Previously, other researchers demonstrated that etoposide induces a decrease in glucose uptake by Hodgkin’s lymphoma cells possible through downregulating glucose transporters [46]; also, there has been described that genotoxic exposure inhibits the expression of both GLUT1 and GLUT3 genes [47]. Similar discoveries were described for Rapamycin, specifically rapamycin reduces glucose uptake in human adipocytes [48], and in CTLs cells rapamycin caused an approximate 50% reduction in levels of GLUT1 and GLUT3 [49].

When we assess the effect of etoposide and rapamycin on cell viability, both treatments induced a significant decrease in this parameter. Our results suggest that etoposide induces an increase in the expression of apoptosis-associated proteins such as FAS and NOXA genes can be part of the molecular mechanism associated with this phenomenon, and in fact, DNA damage induces the expression of FAS in other cancer cells [50]. On the contrary, the treatment with rapamycin induces a clear increase in cell death, and an increase in the levels of cleaved caspase 3 and fragmented PARP, which suggests the initial steps of the apoptotic process.

Both drugs provoke a decrease in proliferative markers, such as Ki67 and PCNA. It is well described that rapamycin can attenuate mammalian cell proliferation, including cancer and immune cells [51]. Inhibition of mTOR in B cells, with rapamycin, suppress cell proliferation by blocking cell cycle progression at G1 phase [52,53]; indeed, mTOR is a crucial regulator of cell cycle progression from G1 to S phase [54].

Summarizing, etoposide and rapamycin have important effects on the metabolic gene expression pattern as well as apoptotic and proliferation markers in these cells. This work provides important evidence since the understanding of cancer metabolism is essential for clinical treatment using those drugs in future.

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**Conflict of interest**

The authors declare no conflict of interest.

**Data accessibility**

The data that support the findings of this study are available from the corresponding author (angara.zambrano@uach.cl) upon reasonable request.

**Author contributions**

MC-G and AZ conceived and designed the experiment; MC-G, YA, CC, CM, PO, GV, and AZ performed experiments and acquired the data; MC-G, CC, MS, FJM, and AZ analyzed and interpreted the data; MC-G, MS, FJM, AR, and AZ drafted, edited, and revised manuscript.

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**Appendix**

**Table A1.** Oligonucleotide primers for real-time RT-qPCR.

| Primers | Sequence 5′-3′ |
|---------|----------------|
| 36B4 Forward | TGGCGGACATCTACAACCTGAAGTT |
| 36B4 Reverse | CAGCCCCGCTCTTGAACAGCACA |
| ACSL3 Forward | ACACAGGGCCTACATATCC |
| ACSL3 Reverse | GGTCGGTGAACAGAAGGCAG |
| BAX Forward | GCCAGCTGACGATTTTTCGAC |
| BAX Reverse | CACCCCAACACCTCTGGTCTT |
| BL2 Forward | TTAGGTTCGGTGCTGTTCAT |
| BL2 Reverse | GACTTCACTTGTTGGCCACAG |
| FAS Forward | AGCTTTGGCTTATAGTGAAGAAA |
| FAS Reverse | GAGGCGAGATCTAGTAGATAT |
| G6PD Forward | AAGACGCTTCTAGTGCCGG |
| G6PD Reverse | AAATAGGGGATGCTGGTCTTTG |
| GLUT1 Forward | CTTACGCTGCTGTCTCCTGTT |
| GLUT1 Reverse | CAGGCCACCACCTTCAAGAAA |
| GLUT2 Forward | GCTATTGCGGCGCTGCTATGG |
| GLUT2 Reverse | CCACACGCGCTGAGAGATCTGC |
| HIF1 Forward | CGTGTGCTTCGAATGTGTC |
| HIF1 Reverse | TACGTTGGGAGGTCTGTAAGTG |
| HK2 Forward | CAAATGAGCAGAGTGTTGGG |
| HK2 Reverse | GCCAGGTCCTCCACTGCTC |

**Table A1.** (Continued).

| Primers | Sequence 5′-3′ |
|---------|----------------|
| KI67 Forward | CGTCCGAGTGGAGAGATTGT |
| KI67 Reverse | CGACCCCCCTCTTTTTGATA |
| LDH Forward | ATCTTGGACCTACCTGTTGGA |
| LDH Reverse | CCATAGCCACCACCTGTAATTG |
| MCM2 Forward | ATCTACGGCAGAGGAGGT |
| MCM2 Reverse | GTAATGGGGATGCTGCCTGT |
| mTAF Forward | AM2GGNTAGGCAACAGAAAAC |
| mTAF Reverse | CAAGTATATGCTGCGAGAAGCT |
| MKV Forward | GCTCAAGTTCCCCAGAGAT |
| MKV Reverse | ATGGTTGGCTGTTATCCTCA |
| NOXA Forward | GTGTTCTCTTTGCGCTTCAT |
| NOXA Reverse | GGAATTTTCGCAACCCTT |
| PCNA Forward | CCGGTTACTGAGGGCGAGA |
| PCNA Reverse | GACAGCCGCTGAGACTTG |
| PDH Forward | GTGATAAATGCTCTCAGAGAGCC |
| PDH Reverse | CAGCACAGTGACAGACAGC |
| PDK1 Forward | GAAGCAGTTCTGCGTTGCTTC |
| PDK1 Reverse | ACCATGAGCGCAGAGAT |
| PFKP Forward | CTACAAAGGCACTTCCACAT |
| PFKP Reverse | ATCATAGATGGCGAGCATCCC |

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1521
| Primers | Sequence 5’–3’ |
|---------|----------------|
| PGD Forward | GGTGCACAACGGGATAGAGT |
| PGD Reverse | CCATCGGTGTCCTGGGAACTTT |
| PUMA Forward | GACCTCAACGCACAGTACGAG |
| PUMA Reverse | AGGAGTCCCATGATGAGATTGT |
| RPE Forward | TGGAAAGGATCTGGGAAGTG |
| RPE Reverse | CTTTGGGTCAAGATCCATA |
| RPIA Forward | CTGGATCGACACCCAGAGAT |
| RPIA Reverse | CGATCACGATGAAGCCGACTA |
| SC5D Forward | TATCTCTTCGCCCATTTTC |
| SC5D Reverse | TGGCTCATTCACCATTTCAAA |
| SCD1 Forward | CCCAGCTGTCAAAGAGAAGGG |
| SCD1 Reverse | CAAGAAAATGGGCAAGAAAC |
| TCL1 Forward | CGATACCAGTCCTCAGACTCCAGTT |
| TCL1 Reverse | AAAGGAGACAGGTGCTGCAAG |