PIM2 inhibition as a rational therapeutic approach in B-cell lymphoma

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ABSTRACT

PIM serine/threonine kinases are overexpressed, translocated or amplified in multiple B-cell lymphoma types. We have explored the frequency and relevance of PIM expression in different B-cell lymphoma types, and investigated whether PIM inhibition could be a rational therapeutic approach. Increased expression of PIM2 was detected in subsets of mantle cell lymphoma (MCL), diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), marginal zone lymphoma-MALT type (MZL-MALT), chronic lymphocytic leukemia (CLL) and nodal marginal zone lymphoma (NMZL) cases. Increased PIM2 protein expression was associated with an aggressive clinical course in ABC-DLBCL patients. Pharmacological and genetic inhibition of PIM2 revealed p4E-BP1(Thr37/46) and p4E-BP1(Ser65) as molecular biomarkers characteristic of PIM2 activity, and indicated the involvement of PIM2 kinase in regulating mTORC1. The simultaneous genetic inhibition of all three PIM kinases induced changes in apoptosis and cell cycle. In conclusion, we show that PIM2 kinase inhibition is a rational approach in DLBCL treatment, identify appropriate biomarkers for pharmacodynamic studies, and provide a new marker for patient stratification.
INTRODUCTION:

Diffuse large B-cell lymphoma (DLBCL) is the most common type of adult non-Hodgkin lymphoma (NHL) (accounting for approximately 40% of all NHL cases)\(^1\). Rather than being a simple entity, it encompasses a constellation of different disorders with varying clinical presentations, molecular pathogenesis and responses to therapy\(^1\). Expression-profile studies have revealed the existence of several DLBCL categories\(^2\), reflecting their origin from discrete B-cell differentiation stages, or the co-regulated expression of transcriptional signatures that reflect features of the cell of origin, molecular pathogenesis or the microenvironment\(^1,3,4\). Three main subtypes can be distinguished on the basis of the cell-of-origin classification: GCB-DLBCL, which express germinal center (GC) genes, ABC-DLBCL (activated B-like DLBCL), with a signature including plasma cell and NF-kB-expressed genes, and primary mediastinal DLBCL\(^5,6\).

The standard first-line therapy for treating DLBCL patients is a combination of chemotherapeutical agents (CHOP: cyclophosphamide, doxorubicin, vincristine and prednisone) with the Rituximab (R) chimeric CD20 monoclonal antibody\(^7\). In spite of this therapy, the disease remains fatal in 30-40% of patients\(^8\). Novel therapeutic opportunities have been proposed based on molecular profiles, suggesting essential regulatory pathways in lymphomas (NF-kB Pathway, B-Cell-Receptor Signaling, BCL2, BCL\(_6\) and tumor microenvironment) as candidate targets\(^9\).

Although there is a considerable amount of information about the molecular pathogenesis of DLBCL, relatively little progress has been made in developing therapies using compounds that target mutated genes or deregulated pathways. The improved knowledge about the molecular pathogenesis of DLBCL is providing opportunities for new approaches in targeted therapy.

PIM genes have been found to be overexpressed, translocated or amplified in different NHL types. The PIM kinases are a family of serine/threonine kinases composed of three members: PIM1, PIM2 and PIM3. They are involved in the phosphorylation and regulation of several proteins that are essential for cell-cycle progression, metabolism and apoptosis, such as
BAD\textsuperscript{10,11}, p21\textsuperscript{12}, p27\textsuperscript{kip1,13}, AKT\textsuperscript{14}, 4E-BP1\textsuperscript{15}, Mdm2\textsuperscript{16} cMyc\textsuperscript{17} and others\textsuperscript{18}. PIM1 collaborates with c-MYC, inducing lymphomagenesis in mice\textsuperscript{19,20}. Overexpression, translocation or amplification of PIM family genes have been described in many human cancers, including B-cell non-Hodgkin’s lymphoma\textsuperscript{21,22,23}, leukemia\textsuperscript{24}, multiple myeloma\textsuperscript{25}, prostate cancer\textsuperscript{26} and pancreatic cancer\textsuperscript{27}. In addition, 38-45\% of patients diagnosed with diffuse large B-cell lymphoma (DLBCL) present somatic mutations in \textit{PIM1} as a result of aberrant somatic hypermutation\textsuperscript{22,28}, while increased PIM1 and PIM2 expression have been shown to distinguish ABC-DLBCL\textsuperscript{3}. These and other observations have encouraged the development of various compounds targeting PIM genes, all of which are effective in the high micromolar range\textsuperscript{29,30,31,32,33}.

The aim of this study is to investigate whether PIM inhibition is a rational therapeutic approach for treating B-cell lymphoma, developing tools for patient stratification at diagnosis or after pharmacodynamic studies.
MATERIAL AND METHODS

Samples
The series included freshly frozen lymph nodes obtained at diagnosis from patients with mantle cell lymphoma (MCL, 24 cases), diffuse large B-cell lymphoma (DLBCL, 22 cases), follicular lymphoma (FL, 23 cases), marginal zone lymphoma MALT-type (MZL-MALT, 15 cases), nodal marginal zone lymphoma (NMZL, 13 cases) and chronic lymphocytic leukemia (CLL, 17 cases). We selected samples in which the tumor comprised at least 80% of the sample. Seven freshly frozen lymph nodes and six freshly frozen reactive tonsils were used as controls. The proportion of B-cells in the reactive samples was estimated by CD20 staining to vary between 35% and 60%. All samples were provided by the CNIO Tumour Bank Unit, in collaboration with the Hospital Virgen de la Salud (Toledo), Hospital 12 de Octubre, Hospital Universitario La Paz and Hospital Gregorio Marañón (Madrid). The study was performed with the approval of the corresponding Ethical Committee of the Centro Nacional de Investigaciones Oncológicas.

Cell lines, cell culture and drugs
Human cell lines derived from DLBCL patients HBL-1, HLY-1, MD-901, OCILY-10, OCILY-3, RIVA, U2932, OCILY-19, SUDHL-4 and SUDHL-6, were cultured in RPMI 1640 (Sigma Chemical, St Louis, MO) supplemented with 10-20% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine (Life Technologies, Inc., Grand Island, NY), 100 mg/ml penicillin/streptomycin (Life Technologies, Inc.), and grown in a humidified atmosphere at 37°C with 5% CO₂. ETP-39010 (4-[6-[(4-fluorophenyl)methylamino]imidazo[2,3-f]pyridazin-3-yl]phenol) was developed by the Experimental Therapeutics Programme of the CNIO (Madrid, Spain)³⁴.

Gene silencing
Small interference RNA (siRNA) against PIM1, PIM2, PIM3 or a non-template control was used to silence PIM kinases in U2932 and RIVA cell lines. Small interference RNAs (siRNA) were purchased as follows: PIM1 (S10527) and PIM2 (s21751) Silencer® Select Validated siRNAs, from Ambion, Applied Biosystems, Carlsbad, CA, USA; PIM2 (L-005359-00-0005) ON-
TARGETplus SMARTpool siRNAs from Dharmacon Inc., Lafayette, CO, USA; PIM3 Stealth Select RNAi™ siRNA (HSS140560) from Invitrogen, Carlsbad, CA, USA; and Silencer® Negative Control (#AM4611) from Ambion, Applied Biosystems. Initial studies with four different siRNAs were performed to select the most efficient for the knockdown of each targeted gene. Concentrations selected for inhibiting each gene were as follows: 100nM for PIM2 and 50nM for PIM1 and PIM3. siRNAs were efficiently introduced into the cell lines by microporation (Microporator MP-100, Digital Bio, Seoul, South Korea). A fluorophore-labeled siRNA (siGLO Red, Dharmacon Inc., Lafayette, CO, USA) was used to estimate the transfection efficiency, which was greater than 95% in all experiments. Twenty-four and 48 hours after transfection, cells were washed in PBS and pelleted for viability, cell-cycle analysis and for protein extraction and immunoblotting.

Viability, apoptosis, cell cycle

Cell viability was measured by the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI), following the manufacturer’s instructions. Distribution of the cell-cycle phase was determined by staining with 50 μg/mL propidium iodide (PI). Evaluation of the early apoptotic populations (Annexin V+/PI) and late apoptotic or secondary necrotic population (Annexin V+/PI+) was performed by Annexin V-APC/PI surface staining according to the manufacturer’s instructions. Cell-cycle and apoptosis assays were analyzed with a FACSCanto flow cytometer (BD). IC$_{50}$ values were calculated using GraphPad Prism v5 (GraphPad Software Inc, La Jolla, CA).

Western blot

For western blot, proteins were extracted using RIPA buffer (Sigma-Aldrich Inc., St Louis, MO) containing protease inhibitor (Roche, Basel, Switzerland). 40 μg of protein were resolved by SDS-PAGE on a 12-15% gel and transferred to nitrocellulose membranes (Millipore, Billerica, MA). Membranes were blocked in 5% bovine serum albumin and incubated with primary antibodies. They were then incubated with goat anti-rabbit IgG, goat anti-mouse IgG or donkey anti-goat IgG secondary antibodies (Alexa 680 nm or Alexa 800 nm, Rockland, Gilbertsville, PA)
and scanned with an Odyssey Infrared System Scanner (LI-COR, Biosciences, Lincoln, NE). Primary antibodies were purchased as follows: BAD (#9292), pBAD(Ser112) (#9291), AKT (#9272), PIM2 (D1D2) (#4730), 4E-BP1 (53H11) (#9644), p4E-BP1(Thr37/46) (#2855), p4E-BP1 (S65) (#9456) from Cell Signaling Technology, Inc., Danvers, MA; PIM1 (12H8) (sc- 13513) from Santa Cruz Biotechnology, Inc., Santa Cruz, CA; pAKT(Ser473) from BD, Franklin Lakes, NJ; and α-tubulin (T-6074) from Sigma-Aldrich.

**Oligonucleotide microarray analysis of gene expression**

Gene-expression profiling was conducted in a series of 114 B-cell non-Hodgkin lymphoma patients (DLBCL, FL, MALT, MCL, CLL and NMZL). Total RNA was extracted from frozen tumor samples using the Trizol reagent (Invitrogen, Carlsbad, CA, USA), and purified and treated with RNase-free DNase I using the RNeasy kit (Qiagen Inc., Valencia, CA). RNA for gene-expression profiling was hybridized on a Whole Human Genome Agilent 4X44K Oligonucleotide Microarray (Agilent Technologies, Inc., Santa Clara, CA). Total RNA extraction and purification, amplification, labeling, hybridization to Agilent 44K Human Whole Genome microarrays and scanning of output image files were performed according to the manufacturer’s instructions. Data were normalized using the Feature Extraction (v.9.0) program. All microarray data are available at the Gene Expression Omnibus (GEO) under accession number GSE32018.

**Gene set enrichment analysis**

The Gene Set Enrichment Analysis (GSEA) tool (http://www.broad.mit.edu/gsea/) was used to explore functional gene sets, allowing the interpretation of complete gene-expression data in relation to PIM1, PIM2 and PIM3 expression. The gene sets co-regulated with PIM1, PIM2 and PIM3 expression were identified using Pearson correlation, with a minimum of 10 and a maximum of 500 genes per gene set being required to qualify them for further analysis. The selected gene sets were compared with the Lymphoma-Enriched BioCarta (LEB) database, which contains selected gene sets from the BioCarta pathways (http://www.biocarta.com/) and previously published B-cell NHL gene signatures. Gene sets with an FDR less than 0.25 were considered significant.
Real-time quantitative PCR

RNA from frozen tissues was extracted according to standard procedures. Expression of mRNA for PIM2 was measured with the use of TaqMan Gene Expression Assays-on-Demand (Applied Biosystems, Foster City, CA). GUSB was used as an endogenous gene. Quantitative reverse transcriptase PCR (RT-PCR) reactions were performed in triplicate with the ABI Prism 7900 Sequence Detector System (Applied Biosystems). Ct values were exported using SDS software (SDS 2.3), and ΔCT and RQ (2-ΔΔCT) were calculated using Stratagene Universal Human Reference RNA (Agilent Technologies, Inc., Santa Clara, CA) as the calibrator.

Tissue microarrays and immunostaining techniques

Immunohistochemical (IHC) expression of PIM2 was assessed using tissue microarrays (TMAs) for an additional subset of 176 DLBCL tumoral samples. Histological criteria used for their diagnosis and classification were those of the World Health Organization Classification1. All cases positively stained for CD20. GC and ABC were subclassified using the algorithm described by Choi and coworkers5. Immunohistochemistry was performed on these TMA sections using PIM2 mouse monoclonal antibody (clone ANI165B) (see Materials and Methods in Supplementary Figure 1). To ensure the reproducibility of this method, we employed straightforward, clear-cut criteria, and cases were scored as positive (1) or negative (0) for PIM2 antibody when distinct, strong nuclear or cytoplasmic expression was present in most of the neoplastic cells. Whole-tissue sections from reactive lymph node, thymus, spleen, and tonsillectomy specimens were used for the analysis of benign lymphoid subpopulations.

Statistics

The Kaplan-Meier method was used to estimate the distributions of overall survival (OS) and progression-free survival (PFS)36. OS was considered as the time from diagnosis to the date of death from any cause or last contact. PFS was calculated from the time of diagnosis to the date of relapse, death or loss. OS and PFS were calculated according to the definition of Cheson et
The log-rank test was used to compare survival distributions. SPSS version 17.0 (SPSS, Chicago, IL) was used for these analyses.

RESULTS

PIM genes are differentially expressed in NHLs

We analyzed the gene-expression profiling data in this series of 114 NHL patients. The data revealed an increased expression of some PIM family members in subsets of MCL, DLBCL, FL, MZL-MALT, CLL and NMZL cases, compared with reactive tonsils and lymph nodes (Figure 1). Thus, 29% (5/17) of CLL patients, 32% (7/22) of DLBCL patients and 29% (7/24) of MCL patients had a high level of PIM1 expression (> 2-fold), whereas the expression of PIM2 was increased (> 2-fold) in 76% (13/17) of CLL, 45% (10/22) of DLBCL, 30% (7/23) of FL, 80% (12/15) of MZL-MALT, 25% (6/24) of MCL and 77% (10/13) of NMZL. No differences (> 2-fold) were observed in PIM3 expression.

Pathways co-regulated with PIM kinases across NHL types

To identify enriched genes sets we conducted gene-set enrichment analysis in the complete series of patients (including DLBCL, FL, MALT, MCL, CLL and NMZL) and controls (lymph nodes and reactive tonsils). We used the Pearson correlation coefficient to identify genes co-regulated with the expression of PIM1, PIM2 or PIM3 genes (Supplementary Table 1). Our results suggest that these genes may have different roles in NHL B-cells (according to the pathways positively correlated with them; Table 1). We found that variation in the level of expression of PIM2 was significantly associated (p < 0.05) with some of the most important pathways in B-cell lymphoma pathogenesis, including cytokine pathways (such as IL2, IL6 and IL3), JAK-STAT, NF-kB, ERK and MAPK pathways, among others (Table 1). PIM3 was negatively correlated with pathways involved in cell proliferation and oncogenesis, such as those of G2 and c-Myc.
Diffuse large B-cell lymphoma

Several lines of evidence suggest that PIM1 and PIM2 may be involved in DLBCL pathogenesis; *PIM1* has been described as a target for aberrant somatic hypermutation in DLBCL\(^{28}\), while *PIM1* and *PIM2* expression distinguish the ABC-DLBCL subtype\(^3\). Consequently, we decided to explore PIM kinases as candidate therapeutic targets for DLBCL treatment, focusing our studies on PIM2.

Data from gene expression analysis revealed that 45% (10/22) of DLBCL patients showed a high level of *PIM2* expression (> 2-fold) relative to that in reactive tonsils and lymph nodes (Figure 1). To validate these results, we examined PIM2 protein expression by IHC in an independent series of 176 homogeneously treated DLBCL patients, 74 of whom had a GC phenotype and 102 an ABC phenotype. In reactive lymphoid tissue, PIM2 expression was restricted to a small subset of germinal center B-cells, mainly centrocytes and plasma cells (Figure 2A, B). In contrast, 23% (41/176) of DLBCL cases exhibited strong nuclear and/or cytoplasmic positivity for PIM2 (Figure 2C, D), although this was more frequent in the ABC-DLBCL subtype (28/102, 27%) than in the GC subtype (13/74, 18%).

In addition, the expression of PIM2 kinase was investigated in the DLBCL cell lines (HBL-1, HLY-1, MD-901, OCILY-10, OCILY-3, RIVA, U2932, OCILY-19, SUDHL-4 and SUDHL-6) using western blot. Cell lines characteristic of the germinal center DLBCL subtype (OCILY-19, SUDHL-4 and SUDHL-6) expressed low levels of PIM2 protein, while ABC-DLBCL cell lines (HBL-1, HLY-1, MD-901, OCILY-10, OCILY-3, RIVA and U2932) showed high levels of expression of PIM2 (\(p = 0.017\); Figure 2E). The expression of PIM2 kinase was also investigated by qRT-PCR, showing a relatively good correlation between mRNA and protein expression (\(p < 0.001\); data not shown).
PIM pharmacological inhibition induces apoptosis and cell-cycle arrest in DLBCL cell lines

Bearing in mind that PIM2 was found highly expressed in a subset of patients and cell lines characteristic of ABC-DLBCL subtype, and taking into account its proposed role in tumor resistance to apoptosis\textsuperscript{39}, we decided to evaluate whether inhibiting PIM2 kinases could have any effect in cell proliferation and survival. Since, to date, no specific inhibitor of PIM2 kinases activity has been developed, we treated ABC-DLBCL cell lines with a small molecule pan-PIM kinase inhibitor (ETP-39010)\textsuperscript{40,34}. The IC\textsubscript{50} determined for the ETP-39010 compound was in the low micromolar range for the DLBCL cell lines (Table 2).

Increased apoptosis was detected after 24-48 h by Annexin V+ and propidium iodide (PI) staining. A time-dependent increase in the population of apoptotic cells was observed in all cell lines (Figure 3A). The OCILY-10 cell line was the most sensitive to PIM inhibitor, showing 48% apoptosis 24 h after inhibition, and 71% after 48 h. The effect of PIM inhibitor on the cell cycle in DLBCL cell lines was also investigated. The cell cycle of DLBCL cell lines treated with ETP-39010 or vehicle alone (DMSO) analyzed at 24 and 48 h, and the cell-cycle distribution was examined by flow cytometry (Figure 3B). G1 cell-cycle arrest was observed in HBL-1 and OCILY-3 cells after 24 h of treatment, and in HBL-1, OCILY-10 and OCILY-3 cells after 48 h of treatment.

Biomarkers of PIM inhibition: AKT, BAD, 4E-BP1

Several substrates of PIM kinase activity have been described in the literature\textsuperscript{3,10,11,12,13,14,16,17}. PIM kinases bind to, phosphorylate and inactivate BAD, both \textit{in vitro} and \textit{in vivo}, on Ser112, a gatekeeper residue for its activation and apoptotic resistance\textsuperscript{10,11}. Treatment of DLBCL cells with 10 \textmu M ETP-39010 for 4 h resulted in a maximum decrease of 20\% in BAD-phosphorylation at Ser112, while the total amount of BAD remained unchanged (Figure 4C). As the reduction in p-BAD levels was less than expected after PIM inhibition, additional PIM substrates were
assayed. We focused our efforts on the study of AKT, first because of the involvement of AKT in BAD phosphorylation and, second, because of the involvement of the PI3K/AKT pathway in regulating PIM1 expression under hormone stimulation and prostate cancer progression14. It has also been reported that PIM1 overexpression increases AKT expression and phospho-AKT (Ser473) levels, indicating the existence of a regulatory feedback mechanism between these two proteins41. Western blot analysis of cell lysates from ETP-39010-treated ABC-DLBCL cells shows that the phosphorylation of AKT at Ser473 was almost completely inhibited relative to the total amount of AKT protein in RIVA and U2932 cell lines, whereas a reduction of about 40% was observed in the remaining three cell lines (Figure 4A). These data suggest that ETP-39010’s mechanism of action involves the regulation of AKT phosphorylation.

Interestingly, recent studies in T-cell lymphomas suggest a role for PIM kinases in modulating the mTORC1 pathway through the phosphorylation of 4E-BP1 at Thr37/46 residue15 as well as the involvement of PIM2 in phosphorylation of 4E-BP1 at Ser65 residue39. Our data extend these observations to DLBCL cells, showing an almost complete inhibition of 4E-BP1 phosphorylation at Thr37/46 and Ser65, 4 h after treatment with the ETP-39010 compound (Figure 4B).

**PIM2 genetic inhibition**

With the intention of refining the analysis and distinguishing whether previously described results were a consequence of inhibiting the highly expressed PIM2 or if a simultaneous inhibition of all three PIM kinases was required, we carried out an independent genetic inhibition of *PIM2*. For this purpose we used two independent sets of small interference RNA against *PIM2* in two ABC-DLBCL cell lines: RIVA and U2932. Western blot analysis of cell lysates demonstrated a 70% downregulation of PIM2 protein levels in the U2932 cell line, whereas a reduction of 40% was observed in the RIVA cell line (Figure 5A). In parallel with the inhibition of PIM2 kinase, a decrease in phosphorylation of 4E-BP1 was observed at two different residues (Ser65 and Thr37/46) in both U2932 and RIVA cell lines (Figure 5A). These results indicate that
PIM2 kinase has an active role in the regulation of the mTORC1 pathway. Its role in the activity of mTORC2 pathway was also examined, by measuring changes in AKT phosphorylation at Ser473. Only a slight inhibition of AKT phosphorylation was observed after PIM2 inhibition, indicating that PIM2 has not a main role in the regulation of mTORC2 pathway in the studied cell lines.

Apoptosis and cell-cycle analysis of DLBCL cell lines was performed 24, 48 and 72 h after microporation. In contrast with findings from ETP-39010 treatment, no effect on apoptosis was detected in RIVA or U2932 cells after PIM2 inhibition. As the ETP-39010 compound is a pan-PIM kinase inhibitor, we decided to check whether a simultaneous inhibition of all three kinases was required. Our results revealed 30% apoptosis in both U2932 and RIVA cell lines 24 and 48 h after simultaneous inhibition of all three kinases (Figure 5B), our results being consistent with those from pharmacological inhibition.

Clinical correlation

There are several lines of evidence associating PIM expression with poor prognosis in most hematopoietic malignancies\(^3,21\). In particular, PIM1 expression has been described as a predictor of poor outcome in mantle cell lymphoma\(^21\). To establish whether differences observed in the intensity of the expression of PIM2 protein were associated with clinical outcome, we used the Kaplan-Meier method to compare high with low or undetectable PIM2 IHC expression in the DLBCL series. Patients who were positive for PIM2 expression appeared to have a shorter OS (\(p = 0.001\)) (Figure 6A). When DLBCL cases were divided into GC and ABC phenotypes, the difference in survival probability was shown to be restricted to those of the ABC-DLBCL subtype (Figure 6B).
DISCUSSION

In the present study, we performed gene-expression analysis of all three PIM kinases in a series of 114 non-Hodgkin lymphoma patients, featuring the most frequent subtypes of B-cell lymphoma (DLBCL, FL, MALT, MCL, NMZL and CLL). Our results reveal subsets of patients with high expression levels of \(PIM1\) (CLL, DLBCL and MCL) and \(PIM2\) (CLL, DLBCL, FL, MALT, MCL and NMZL) (Figure 1), thus confirming previous suggestions of a pathogenic role for PIM kinases in different non-Hodgkin lymphoma subtypes (DLBCL, MCL, etc.)\(^3,28,21,42\). With the intention of discerning whether these differences were associated with any particular biological role, we examined whether the \(PIM\) family gene expression pattern was co-regulated with gene sets or functional pathways.

The results showed \(PIM2\) expression to be associated with B-cell survival pathways such as those involving cytokines (IL6, IL10 and IL3), and CD-40, NF-kB and p53 signaling. Consistent with previously published data, \(PIM1\) was found to be positively correlated with the p53\(^16\), FAS and caspase pathways. \(PIM3\) expression exhibited mainly negative associations (with c-Myc and BLIMP-1 targets, and G1 and cell-cycle pathways), and a positive association with the KRAS signature. Although it has been hypothesized that \(PIM1\), \(PIM2\) and \(PIM3\) have some functional redundancy\(^20,43,44\), very few enriched gene sets were shared by PIM family members. In fact, pathways associated with the \(PIM3\) signature differ completely from those associated with \(PIM1\) and \(PIM2\), suggesting dissimilar roles, at least in B-cell lymphoma.

The expression of PIM genes is induced by multiple cytokines through the activation of JAK/STAT signaling pathways\(^45,12\). Recent findings have implicated PIM1 in the regulation of SOCS-1, a negative regulator of the JAK/STAT pathway, and suggested that PIM1 can also modulate cytokine signaling pathways in addition to its role as a direct effector kinase\(^26\). Our findings show that, in B-cell lymphomas, \(PIM1\) and \(PIM2\) are positively co-regulated with the JAK/STAT pathway, whereas \(PIM3\) has no such association. However, slight differences were observed among JAK/STAT genes co-regulated with \(PIM1\) and \(PIM2\). Although \(PIM2\) was co-
regulated with PIAS3, PTPN6, and PTPRC pathway inhibitors, PIM1 was co-regulated with SOCS-1 (Supplementary table1). Both genes were positively associated with different JAK/STAT genes (JAK1, JAK2, STAT1, STAT3), but PIM2 was also associated with JAK3, STAT5A and IL6ST.

The discovery of PIM serine/threonine kinase involvement in development and progression of several cancers has made PIM kinases the object of pharmaceutical interest42, and some chemical compounds or specific antibodies have already been found to have an inhibitory effect on them29,30,31,32,33. Given the existence of PIM1 somatic mutations in DLBCL28 and the increased expression of PIM1 and PIM2 in the ABC-DLBCL subtype3, we decided to focus our studies on DLBCL cases and cells, using genetic inhibition and a novel pan-PIM kinase inhibitor (ETP-39010) in parallel40. Our results reveal that the inhibition of PIM kinases is cytotoxic for DLBCL cell lines, and that toxicity is time-dependent (Figure 3A). Moreover, addition of the PIM-inhibitor ETP-39010 induces G1 cell-cycle arrest in HBL-1, OCILY-10 and OCILY-3 cells. The genetic inhibition studies described here reveal that induction of apoptosis in the RIVA and U2932 cell lines requires the simultaneous inhibition of PIM1, PIM2 and PIM3. In fact, PIM3 has a central role in the induction of apoptosis in the RIVA cell line. The possibility of overlapping substrates and the suggestion of additive effects20 of the different PIM family members as demonstrated by the triple knockout46 indicates that the inhibition of all isoforms may be more effective than targeting individual isoforms. Nevertheless, it is a concern that PIM kinase inhibition may be generally toxic and not highly preferential to the tumor cells, leading to substantial side-effects on normal cells. However, the lack of any overt phenotypes in Pim1-/-; Pim2-/-; Pim3-/- mice indicates that PIM inhibition might not have side-effects on normal B-cells46.

The identification of molecular biomarkers characteristic of PIM activity is one of our areas of interest. Several such biomarkers have been described in the literature, including cell-cycle regulators (p21\textsuperscript{CIP1/WAF1,12}, CdC25C\textsuperscript{47}, C-TAK1\textsuperscript{48}, p27\textsuperscript{KIP,13}), proteins involved in proliferation and
cell survival (BAD\textsuperscript{10}) and those regulating signaling pathways (SOCS\textsuperscript{29}, COT (IKKB)\textsuperscript{49}, AKT\textsuperscript{32}). Although phosphorylation of BAD at Ser 112 is one of the main targets of PIM kinases, our results show a weak response to PIM inhibition, with a maximum inhibition of 20%. This result suggests either the existence of additional targets of major importance in DLBCL or the presence of proteins with a similar substrate. Accordingly, we decided to study phosphorylation at two additional targets: AKT on Ser473 and 4E-BP1 on the Thr37/46 and Ser65 residues. AKT is a protein with a possibly redundant role in regulating cellular proliferation and survival, due to the similar substrate specificities shared by PIM1 and AKT\textsuperscript{41}. Recent studies have suggested that PIM kinases help to modulate the mTORC1 pathway by phosphorylating 4E-BP1 at the Thr37/46 residue while, conversely, they have no effect on the mTORC2 substrate AKT on Ser473\textsuperscript{15}. Our findings show that both pathways are regulated by PIM kinases in DLBCL cell lines, since reduced phosphorylation of 4E-BP1 at Thr37/46 and of AKT at Ser473 was observed 4 h after treatment with the ETP-39010 compound. Moreover, an almost complete inhibition of phosphorylation of 4E-BP1 at the Ser65 residue was observed in all the studied DLBCL cell lines. The independent genetic inhibition of PIM2 confirmed its involvement in the phosphorylation of 4EBP1 on either Ser65 or Thr37/46 residue, supporting 4EBP1 phosphorylation as an optimal biomarker of PIM2 inhibition. Despite the strong reduction of AKT phosphorylation observed with the ETP-39010 compound, no inhibition was noticed after PIM2 gene knockdown, meaning that results obtained after pharmacological treatment can either be a consequence of PIM1 inhibition\textsuperscript{32} or due to the existence of other targets for the compound used here\textsuperscript{50}.

Diffuse large B-cell lymphoma is the most frequent form of aggressive lymphoma, and accounts for 30-40% of newly diagnosed lymphomas. The standard therapy used for these patients (combination chemotherapy plus Rituximab, R-CHOP treatment) can cure 55-60% of cases. It has also been shown that patients with the GCB subtype have favorable OS rates after R-CHOP treatment compared with those of the ABC subtype\textsuperscript{9}. In the series reported here, we found that
the expression of PIM2 protein distinguishes a group of ABC-DLBCL cases with aggressive behavior after R-CHOP treatment, a group of patients who are candidates for pharmacological PIM inhibition (Figure 6). The characteristics of these cases did not fully coincide with those described as STAT3+ DLBCL, a subset of tumors that also display aggressive behavior\(^3\), since there was no direct correlation between PIM2 and STAT3 expression (Pearson correlation coefficient 0.2; p > 0.01; data not shown).

The identification of novel potential therapeutic targets provides new opportunities for the treatment of aggressive DLBCL cases. In this study, we show that PIM2 inhibition is a rational approach to DLBCL treatment, and can be accomplished through genetic or pharmacological inhibition. The study identifies also markers for patient stratification (PIM expression) and pharmacodynamic studies (p4EBP1). Our findings also confirm that PIM2 regulates the expression of 4E-BP1, a pharmacodynamic marker of mTOR activity, suggesting that PIM2 is involved in regulating cell metabolism\(^4\).
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AUTHORSHIP CONTRIBUTIONS

CGA contributed to the conception and design of the study, performed experiments, interpreted data and wrote the manuscript; HP performed experiments; CBA, JFL and JRB contributed vital reagents; GR contributed to monoclonal antibody development; AGM and GMG evaluated TMAs; JAMC provided samples; EM and MER performed experiments; MSB revised the manuscript; MAP contributed to the conception and design of the study, interpreted data and wrote the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest
REFERENCES


# TABLES

### Table 1. Pathways co-regulated with PIM kinases among B-cell NHL types.

Values are of the significance of FDR.

<table>
<thead>
<tr>
<th>Pathways</th>
<th>PIM1</th>
<th>PIM2</th>
<th>PIM3</th>
</tr>
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<tbody>
<tr>
<td><strong>Positive</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>IL2RB PATHWAY</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>JAKSTAT PATHWAY</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>JAKSTAT RECEPTORS</td>
<td>-</td>
<td>0.076</td>
<td>-</td>
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<tr>
<td>IL2 PATHWAY</td>
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<td>0.076</td>
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<tr>
<td>P38 MAPK PATHWAY</td>
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<td>-</td>
</tr>
<tr>
<td>BM PLASMA CELL</td>
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<td>-</td>
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<td>-</td>
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<tr>
<td>CD40 SIGNALLING DURING GC DEV</td>
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<tr>
<td>Bcells_IgM_IgD_CD27+</td>
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<td>IL6PATHWAY</td>
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<tr>
<td>BLOOD MONOCYTE</td>
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<td>-</td>
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<tr>
<td><strong>Negative</strong></td>
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<td>BLIMP-1 TARGETS</td>
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<td>CK1 PATHWAY</td>
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<tr>
<td>CREBPATHWAY</td>
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We conducted gene-set enrichment analysis in the complete series of patients (including DLBCL, FL, MALT, MCL, CLL and NMZL) and controls (lymph nodes and reactive tonsils). We used the Pearson correlation coefficient to identify pathways co-regulated with the expression of either PIM1, PIM2 or PIM3 genes. PIM2 expression was significantly associated with a larger series of significant gene sets.
Table 2.- Sensitivity to PIM pharmacological inhibition in a panel of DLBCL cell lines. The IC\textsubscript{50} was calculated for each of the DLBCL human cell lines and is shown in micromoles. DLBCL cell lines were treated with 0 - 100 μM ETP-39010 compound for 72 h and assayed for cell viability by MTT assay.

<table>
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<tr>
<th>Line</th>
<th>IC\textsubscript{50}, μM ETP-39010</th>
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<tr>
<td>DOHH-2</td>
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<td>SUDHL-6</td>
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<tr>
<td>OCILY-10</td>
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<tr>
<td>OCILY-3</td>
<td>1.61</td>
</tr>
<tr>
<td>RIVA</td>
<td>1.6</td>
</tr>
<tr>
<td>U2932</td>
<td>4.9</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1.- PIM genes are differentially expressed in B-cell non-Hodgkin lymphoma cases.
A) Heatmap showing PIM kinase gene expression of the complete series of 114 NHL patients and 14 control tissues. Data were normalized with respect to reactive lymph node and tonsils; B) PIM expression in various B-cell lymphoma types. Gene-expression data were normalized with respect to lymph node expression. Log2 ratios are shown and the mean of the series is included for each lymphoma type. There is an increase in the expression of PIM1 and PIM2 in subsets of most lymphoma types.

Figure 2.- PIM2 expression in human DLBCL samples and cell lines. PIM2 immunohistochemical staining of reactive tonsil: original magnification (A), and x100 (B); IHC staining of DLBCL tissues: negative (C) and positive (D) PIM2 staining. Western blot analysis (E) and qRT-PCR (F) of PIM2, in a panel of DLBCL cell lines characteristics of germinal center (GC) or post-germinal center. The expression of PIM2 is significantly higher (t-test, *p < 0.02) in cell lines of the ABC-DLBCL subtype. The levels of PIM2 were quantified from the Western blot by using Image J software and normalized against α-tubulin. Data are representative of two independent experiments. RQ (2-ΔΔCT) was calculated using Stratagene Universal Human Reference RNA as the calibrator. RT-PCR reactions were performed in triplicate.

Figure 3.- Inhibition of PIM kinases induces apoptosis and cell-cycle arrest in DLBCL cell lines. A) Induction of apoptosis after treatment for 24 and 48 h with the ETP-39010 compound, at 10μM. The experiment was carried out in triplicate and SD from the mean was represented with error bars. Statistical comparison of treated (ETP-39010) and untreated (DMSO) cells is indicated (*p<0.05; **p<0.001) B) Cell-cycle analysis of DLBCL cell lines treated with ETP-39010 (green line) or vehicle alone (DMSO; red line) was performed for 24 and 48 h. Cells were
stained with propidium iodide and analyzed for cell-cycle distribution by flow cytometry. HBL-1, OCILY-10 and OCILY-3 cells were arrested in G1 phase and underwent time-dependent apoptosis. The experiment was carried out in triplicate and SD from the mean was represented between brackets.

**Figure 4.- Biomarkers of PIM inhibition.** DLBCL cells were incubated for 4 h with ETP-39010 (10 µM) or vehicle DMSO alone (represented as ETP and C respectively). A,B) Cellular levels of AKT, pAKT (Ser473), 4E-BP1 and p4E-BP1(Thr37/65) were detected by western blot using antibodies for these proteins and tubulin as the loading control. Bands were quantified using the ImageJ program and the ratios of phosphorylated to total protein were presented. The blot is representative of two independent experiments. C) Cellular levels of pBAD (Ser112) and BAD were measured by flow cytometry and the ratios of phosphorylated to total protein were presented (each column represents ratios from triplicate experiments).

**Figure 5.- PIM2 knockdown: biomarkers and effects on apoptosis and cell cycle.** A) Western blot analysis of PIM2, p4E-BP1(Thr37/46) and p4E-BP1(Ser65), in RIVA and U2932 cell lines. Knockdown inhibition of PIM2 was achieved with two independent sets of small interference RNA: siRNA1 (L-005359-00-0005) and siRNA2 (s21751). siRNAs with sequences that do not target any gene product (scramble) were used as controls. Bands were quantified using the ImageJ program and the ratio of phosphorylated to total protein was calculated. The blot is representative of two independent experiments. B) Induction of apoptosis and cell-cycle were measured 24 h and 48h after knockdown inhibition of PIM1, PIM2 (siRNA1 and siRNA2), PIM3, or the simultaneous inhibition of all three PIM family members using either PIM2-siRNA1 or PIM2-siRNA2 (T-siRNA1 or T-siRNA2, respectively). Scrambled siRNAs were used as controls. No effects on cell cycle were observed at any time or with any siRNA combination (See Supplementary table 2 for quantification of cell cycle data).
Figure 6.- **PIM2 is associated with shorter overall survival and progression-free survival in ABC-DLBCL patients.** Kaplan-Meier survival curves of the entire series of 176 DLBCL patients, analyzed as a group (A) or separately by its main subtypes (B): ABC-DLBCL and GC-DLBCL; PIM2-positive cases are represented in green; negative cases are shown in blue. Kaplan-Meier analysis and the log-rank test gave significant results for overall survival and progression-free survival relative to the ABC-DLBCL subtype.
Figure 1
Figure 2
Figure 3

A) Apoptosis (ETP-39010)

% Annexin V+

HBL-1  OCILY-10  RIVA  U2932  OCILY-3

24h  48h

B) 24h  48h

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>24h</th>
<th>48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBL-1</td>
<td>56.8%±(0.9)</td>
<td>83.7%±(1.7)</td>
</tr>
<tr>
<td>OCILY-10</td>
<td>25.1%±(4.8)</td>
<td>6.0%±(0.9)</td>
</tr>
<tr>
<td>RIVA</td>
<td>13.9%±(3.8)</td>
<td>5.8%±(0.6)</td>
</tr>
<tr>
<td>U2932</td>
<td>70.8%±(0.5)</td>
<td>78.7%±(4.9)</td>
</tr>
<tr>
<td>OCILY-3</td>
<td>13.8%±(0.64)</td>
<td>6.0%±(0.22)</td>
</tr>
<tr>
<td></td>
<td>10.1%±(0.33)</td>
<td>6.5%±(1.4)</td>
</tr>
</tbody>
</table>

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Figure 4

A) pAKT(Ser473)

B) p4E-BP1(Thr37/46)

C) p-BAD (Ser112)
Figure 5
A) Total-DLBCL
Survival probability

- p = 0.001; N = 176

B) GC-DLBCL
Survival probability

- p > 0.05; N = 74

AB-DLBCL
Survival probability

- p = 0.001; N = 102

Figure 6
PIM2 inhibition as a rational therapeutic approach in B-cell lymphoma

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