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Resistance to PI3Kδ inhibitors in marginal zone lymphoma can be reverted by targeting the IL-6/PDGFRA axis

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Author contribution
AJA and SN equally contributed. AJA performed experiments, analyzed and interpret data, performed data mining, prepared the figures and cowrote the manuscript; SN performed silencing experiments and interpreted data; LC performed data mining; GS, EG, CT, AM, FS, LB performed experiments; AZ, FR, RBP, GS, VG performed flow-cytometry analyses; AR performed genomics experiments; MCM, ME performed methylation profiling experiments and data mining, SJ performed ELISA Luminex experiments; AS provided advice, JRB collected and characterized tumor samples; EZ and DR codesigned research, edited the manuscript, FB designed research, interpreted data, and cowrote the manuscript; all authors approved the final manuscript. AJA and FB are co-corresponding authors.
Conflict of interests

Alberto J. Arribas: travel grant from Astra Zeneca.
Luciano Cascione: travel grant from HTG.
Laura Barnabei: currently part-time employee of Bright Peak Therapeutics.
Anastasios Stathis: institutional research funds from: Bayer, ImmunoGen, Merck, Pfizer, Novartis, Roche, MEI Pharma, ADC-Therapeutics; travel grant from AbbVie and PharmaMar.
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The other Authors have nothing to disclose.

Data sharing statement
Genomics data will be available in GEO database (under submission).

Statement of translational relevance
Multiple PI3Kδ inhibitors are approved and/or under clinical investigation for B-cell lymphoid neoplasms. The development of a novel model of secondary resistance derived from splenic marginal zone lymphoma shows that therapeutic approaches targeting IL-6/STAT3, PDGFRA, CD19, and LIN28 might improve the responses PI3Kδ inhibitors.
Abstract

PI3Kδ inhibitors are active in patients with lymphoid neoplasms and a first series of them have been approved for the treatment of multiple types of B-cell lymphoid tumors, including marginal zone lymphoma (MZL). The identification of the mechanisms underlying either primary or secondary resistance is fundamental to optimize the use of novel drugs. Here, we present a model of secondary resistance to PI3Kδ inhibitors obtained by prolonged exposure of a splenic MZL cell line to idelalisib. The VL51 cell line was kept under continuous exposure to idelalisib. The study included detailed characterization of the model, pharmacological screens, silencing experiments, validation experiments on multiple cell lines and on clinical specimens. VL51 developed resistance to idelalisib, copanlisib, duvelisib, and umbralisib. An integrative analysis of transcriptome and methylation underlined an enrichment of up-regulated transcripts and low-methylated promoters in resistant cells, including IL-6/STAT3 and PDGFRA related genes and surface CD19 expression, alongside the repression of the let-7 family miRNAs, of miR-125, miR-130, miR-193 and miR-20. The use of the IL-6R blocking antibody tocilizumab, the STAT3 inhibitor stattic, the LIN28 inhibitor LIN1632, the PDGFR inhibitor masitinib and the anti-CD19 antibody drug conjugate loncastuximab tesirine were active compounds in the resistant cells as single agents and/or in combination with PI3Kδ inhibition. Findings were validated on additional in vitro lymphoma models and on clinical specimens. A novel model of resistance obtained from splenic MZL allowed the identification of therapeutic approaches able to improve the anti-tumor activity of PI3Kδ inhibitors in B-cell lymphoid tumors.
Introduction

PI3Kδ shows prominent expression across hematopoietic tissues and has a central role in B cell receptor (BCR) signaling (1, 2). Indeed, its inhibition is being extensively explored as a therapeutic approach for patients with lymphoid neoplasms (1-3). Idelalisib was the first-in-class specific PI3Kδ inhibitor to show clinical activity as single agent in patients with follicular lymphoma (FL), marginal zone lymphoma (MZL), chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL), and in combination with rituximab in CLL patients (1, 2), and it received the approval by the U.S. Food and Drug Administration (FDA) for the treatment of patients with relapsed follicular lymphoma (FL) or small lymphocytic lymphoma (SLL) after at least two prior systemic therapies, and, in combination with rituximab, for patients with relapsed chronic lymphocytic leukemia (CLL) (3). Idelalisib has been successfully followed by a series of second generation PI3Kδ inhibitors, such as parsaclisib or zandelisib, and by compounds that inhibit additional kinases, such as copanlisib (PI3Kα/PI3Kδ), duvelisib (PI3Kδ/PI3Kγ) or umbralisib (PI3Kδ and casein kinase-1ε), achieving clinical responses in lymphomas, including FL, MCL, MZL and CLL (1-3). In particular, data have so far led to the FDA approval of copanlisib for relapsed/refractory FL patients, duvelisib for patients with relapsed/refractory CLL, SLL or FL, and umbralisib for the treatment of relapsed/refractory MZL or FL patients (3). The identification of the mechanisms underlying either primary or secondary resistance to PI3Kδ inhibitors is fundamental to optimize the use of these drugs, and a number of studies have described mechanisms of resistance to this class of agents in lymphoid neoplasms, driven by activation of alternative signaling cascades (4-11). Here, we present a model of secondary resistance to PI3Kδ inhibitors, including duvelisib, copanlisib and umbralisib, obtained by prolonged exposure of a splenic MZL cell line to idelalisib.

Methods

Development of resistant cell lines
VL51 cells were cultured according to the recommended conditions, as previously described (12). To develop resistance cell lines were exposed to IC90 concentration of idelalisib (Selleckchem, TX, USA) for several months until they acquired specific drug resistance (resistant). In parallel, cells were cultured upon similar conditions in the absence of drug (parental). Proliferation of stable resistance was tested by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay after 2-weeks of drug-free culture. Multi-drug resistance phenotype was assessed by real-time PCR for MDR1 and MDR2-3 genes using published primers (13). We developed biological replicates by splitting the resistant clones after one month from resistance development, keeping them separate for six months before performing further experiments. Cell line identity was periodically authenticated by short tandem repeat (STR) DNA profiling, as previously described (14). Cells were periodically tested to confirm Mycoplasma negativity using the MycoAlert Mycoplasma Detection Kit (Lonza, Visp, Switzerland).

Treatments
Response to single or drug combination treatments was assessed upon 72hr of exposure to increasing doses of drug followed by MTT assay. Copanlisib, duvelisib, umbralisib, everolimus, bimiralisib, vincristine, decitabine, masitinib, statric and tocilizumab were purchased from Selleckchem, Lin28-1632 (LIN1632) from R&D Systems (Minneapolis, MN, USA), human recombinant IL-6 (CYT-213) from Prospec (Rehovot, Israel). Loncastuximab tesirine was kindly provided by ADC Therapeutics (Epalinges, Switzerland). See details in Supplementary methods.

Genomics and Data mining
See Supplementary methods.

ELISA
For ELISA assays the conditioned medium was cultured for 72h and collected. Then medium was filtered twice (22 µm) and centrifuged at 4000 rpm for 30 minutes in Amicon Ultra-4 tubes (Ultracel 3k, Merk Millipore) to remove cells and particles, and analyzed via cytokine array or ELISA. Cytokine arrays (Human Cytokine Array Panel A, R&D Systems) was performed according to the manufacturer’s protocols. ELISA assays on frozen human serum samples were performed using Luminex Assay (R&D Systems) according to the manufacturer’s protocols. The serum samples were collected from patients treated with idelalisib and enrolled on tissue banking protocols at DFCI; all patients signed written informed consent prior to a sample being drawn, and the protocols were approved by the Dana-Farber Harvard Cancer Center Institutional Review Board.

Flow Cytometry and protein analyses
Flow Cytometry was performed to determine the surface expression of PDGFRA, IL-6R, IL-6ST, CXCR4 and CD19...
(Table S1), levels of p-AKT, p-BTK, p-PLCG2, p-mTOR and p-ERK (Table S2); and cell cycle phases. Immunoblotting was performed to determine the expression of AKT/p-AKT, ERK/p-ERK JAK/p-JAK, STAT/p-STAT and GAPDH (Table S3). See details in Supplementary methods.

**Pharmacological combination screening**

Pharmacological screening was performed by exposing idelalisib-resistant in parallel with parental clones, as a single or in combination with idelalisib, to 348 compounds from a custom library containing agents belonging to the following classes: “Kinase Inhibitory”, “Epigenetic Compound”, “PI3K/Akt Inhibitor”, “Apoptosis”, “Anti-cancer Compound”, and “MAPK Inhibitor” (Selleckchem). Cells were treated with library (1µM) as a single or in combination with 1µM of idelalisib. Cell viability was measured after 72h exposure by MTT assay.

**Gene silencing**

See Supplementary methods.

**Results**

A splenic MZL model of secondary resistance to the PI3Kδ inhibitors

To create a novel model of resistance to the PI3Kδ inhibitors, we selected the splenic MZL-derived VL51 cell line, with transcriptome, somatic mutational status, DNA profile and methylome comparable to primary splenic MZL samples (15, 16). VL51 harbors a series of lesions that are typical of splenic MZL, including truncating mutations of NOTCH2 and BIRC3, which activate downstream NOTCH and non-canonical NF-κB, respectively, and a nuclear localization signal mutation of KLF2 (15, 17, 18).

After six months of continuous exposure to the FDA approved PI3Kδ inhibitor idelalisib, VL51 developed a stable resistance with an IC50 25-fold higher than its parental counterpart (Fig 1A). The resistance was confirmed to be stable repeating the IC50 after two weeks in medium containing no drug, and multidrug resistance was ruled out by demonstrating no changes in the expression levels of MDR1/2 genes by semiquantitative real-time PCR (Fig S1).

Idelalisib-resistant cell line showed resistance also against other FDA approved PI3K inhibitors, including the PI3Kα/PI3Kδ inhibitor copanlisib, the PI3Kδ/PI3Kγ inhibitor duvelisib, and the PI3Kδ/CK1ε inhibitor umbralisib (Fig 1B-D). Conversely, they maintained sensitivity to the mTOR inhibitor everolimus, to the dual PI3K/mTOR inhibitor bimiralisib (Fig 1E-F) and to the chemotherapy agent and MDR substrate vincristine (Fig 1G).

Resistant cells clearly differed from their parental counterparts based on RNA-Seq (total RNA, miRNA) and methylation, as shown by unsupervised analyses and at the pharmacological profiling (Fig 1, S2, S11), but no exonic nonsynonymous variants appeared acquired in the resistant cells. Table S4 contains the single and copy number variants identified by WES in resistant when compared with parental cells.

Resistance to idelalisib is driven by IL-6 and PDGFRA

Hence, to investigate the mechanism of resistance, we integrated the gene expression (GEP, RNA-seq), miRNA (RNA-seq) and methylation (800k Illumina array) profiles obtained in resistant and parental cells (Table S4). When compared to its parental counterpart, the resistant cell line presented an enrichment in BCR/TLR/NF-κB (TLR4, CD19, SYK), IL-6/STAT3 (IL-6, CD44), chemokines (CXCL10, CXCR4, CXCR3), PDGFR (PDGFRA, PRKCE), IGF1R and RAS-RAF signaling pathways, epigenetic signatures (PRC2-complex targets and methylated genes in cancer) and genes up-regulated in MZLs. On the other hand, it presented a decreased expression of transcripts involved in aminoacid depravation, antigen processing, drug metabolism, translation, proteosome and hypoxia (Fig S2B, S3, Table S5). The integrative analysis of transcriptome and methylation highlighted an enrichment of up-regulated transcripts among the low-methylated promoters in resistant cells, including IL-6 and RAS-related genes (Fig 2, S3). Analysis of the miRNA profiles identified repression of members of the let-7 family (let-7e, let-7g, let-7d, let-7i, let-7a), along with other miRNAs including miR-125, miR-130, miR-193 and miR-20. Conversely, only one member of the let-7 family (let-7c) was among the up-regulated miRNAs in resistant cells, together with miR-3196, miR-4492, miR-4516 and others (Table S5). MiRNAs potentially targeting the IL-6-PDGFR axis were enriched among the repressed miRNAs in resistant cells. Integration of methylation and miRNA data identified fully methylated and repressed miRNAs known to target up-regulated genes: members of let-7 family of miRNAs (let-7d, let-7e, let-7g) that directly target IL-6 (19) miR-125a that regulates IL-6, IL-6R and STAT3 (20), and the negative regulators of PDGFRA signaling: miR-130a (21), miR-193b, miR-20b and miR-17 (22, 23) (Fig 2, Table S5). These data indicated that an epigenetic reprogramming could sustain the observed resistance.

Having observed an up-regulation of the secreted factor IL-6 at RNA-level in resistant cells, we evaluated whether the
transfer of the conditioned media to the parental cells was able to transfer the resistance to PI3K inhibitors. The media from resistant cells (taken at 48 h), but not from media taken from other cells, including parental, gave resistance (Fig 3A). The secretion of IL-6 was confirmed by ELISA (Fig S4), and the over-expression of surface PDGFRA, CXCR4 and CD19 expression by flow-cytometry (Fig 3B, S5). Increased levels of p-ERK (immunoblot and phosphoFlow) and p-STAT (immunoblot) were observed in resistant cells (Fig 3C, S6, S7).

While silencing of the individual genes had only a partial effect, the concomitant silencing of both IL-6 and PDGFRA by siRNAs reverted the resistance (Fig 4A, S8). Consistent with the effect of IL-6 silencing, exposure of parental cells to recombinant IL-6 induced resistance (Fig 4B).

Targeting IL-6/STAT3, LIN28, or PDGFRA reverts the resistance to idelalisib

We then explored pharmacological approaches to overcome the acquired resistance. Based on IL-6 involvement we tested tocilizumab, an IL-6R blocking antibody FDA approved for the treatment of various autoimmune disorders and of cytokine release syndrome (CSR) (24). The addition of tocilizumab to idelalisib overcame the resistance increasing the potency (i.e., the minimal active concentration) of the small molecule in the resistant cells (Fig 4B). No advantage was given by adding the antibody in parental cells. Tocilizumab as single agent showed limited cytotoxicity in either parental or resistant cells (Fig S9). To investigate the signaling activation in resistant downstream of IL-6, we combined idelalisib with the STAT3 inhibitor stattic. Consistent with the involvement of IL-6/STAT3 activation in resistant, and similarly to tocilizumab, addition of stattic decreased cell viability and enhanced sensitivity to idelalisib in resistant, with minimal effect in parental (Fig S11B).

A screening with 348 anti-cancer agents and compounds targeting important biologic pathways identified the acquired sensitivity of the resistant cells to the PDGFR inhibitor masitinib (Fig S12). The efficacy of idelalisib was enhanced by the presence of masitinib (500nM) only in resistant and not in parental (Fig 5B, Fig S13).

CD19 upregulation in resistant cells determines increased sensitivity to CD19 targeting agents

Due to the increased CD19 (RNA and surface) expression in the idelalisib-resistant VL51 cell line compared to its parental counterpart (Fig 6A, 3B bottom, S5E), we evaluated a CD19 targeting treatment, namely the CD19-directed antibody-drug conjugate loncastuximab tesirine (ADCT-402), recently FDA approved for the treatment of relapsed/refractory large B-cell lymphoma patients (25). The idelalisib-resistant cells were much more sensitive than their parental counterpart (Fig 6B).

The detected mechanisms of resistance are not limited to idelalisib nor to the VL51 model

We then investigated whether the mechanism of resistance identified might affect the sensitivity to other PI3K inhibitors, such as duvelisib, umbralisib and copanlisib. Addition of the IL-6R blocking antibody tocilizumab, the STAT3 inhibitor stattic, the PDGFR inhibitor masitinib or the LIN28 inhibitor LIN1632 improved the anti-lymphoma activity not only of idelalisib but also of the other clinically relevant PI3K inhibitors in resistant but not in parental VL51 (Fig S14A-B). Conversely, as observed for idelalisib in parental cells, stimulation with recombinant IL-6 decreased sensitivity to duvelisib, umbralisib and copanlisib, which was restored by the addition of tocilizumab (Fig S14C-D).

To further extend the significance of our findings beyond the VL51 model, we first took advantage of a large series of lymphoma cell lines we had previously characterized at transcriptome level and for their sensitivity to idelalisib (26). IL-6, PDGFR and LIN28 expression levels were inversely correlated with idelalisib sensitivity, whilst the latter was positively correlated with let-7 and miR-125 levels also in these additional B-cell lymphoma models (P<0.05, Fig 7). Second, we selected two B-cell lymphoma models, another splenic MZL SSK41 and the diffuse large B-cell lymphoma-derived RCK8, based on their low sensitivity to idelalisib and their expression of IL-6R, PDGFR and LIN28. We tested the response to idelalisib, duvelisib, umbralisib and copanlisib in combination with the corresponding inhibitors tocilizumab (anti-IL-6R), masitinib (PDGFR inhibitor) and LIN1632 (LIN28 inhibitor). In line with what observed in the VL51 model, both SSK41 and RCK8 benefited by the addition of tocilizumab, masitinib and LIN1632 (Fig S15A-B).
the sensitivity to idelalisib and to the additional PI3K inhibitors duvelisib, umbralisib and copanlisib was evaluated in primary idelalisib-sensitive B-cell lymphoma cell lines with expression of \textit{IL-6R}, including the mantle cell lymphoma models Granta519 and JVM2. Similarly to VL51 parental cells, stimulation with recombinant IL-6 decreased sensitivity to all PI3K inhibitors, and addition of tocilizumab recovered response to the drugs (Fig S15C-D).

**Exposure to epigenetic drugs improves sensitivity to idelalisib**

Based on the different epigenetic profiles observed in parental and resistant cells, we evaluated whether resistance may be reverted using epigenetic drugs. Due to the enrichment in resistant cells for targets of PRC2-complex and methylated genes in cancer (Fig S2B), we tested the combination of idelalisib with the EZH2 inhibitor tazemetostat and the demethylating agent 5-azacitidine (5-Aza). Resistant and parental cells were exposed to 5-azacitidine (5-Aza) or to tazemetostat given concomitantly to idelalisib or five days before the PI3K inhibitor. Concomitant combination exhibited very limited effect in either parental or resistant (Fig S16A). Nevertheless, and consistent with a mechanistic role of methylation in the resistance to idelalisib, pre-treatment with either 5-Aza (200 nM) or tazemetostat (1 µM) was beneficial in resistant and not in parental (Fig S16B).

**The factors associated with resistance in cell lines are also relevant in clinical specimens**

To extrapolate our findings to the clinical context, we studied both available expression datasets and a series of serum samples. PDGFRA, IL-6 and IL-6 receptor appeared expressed in two series of retrospectively collection of MZL clinical specimens and in a large series of DLBCLs (27-29) (Fig S17A-D). Taking advantage of the previously reported gene expression profile of splenic MZL clinical specimens (28), we determined the top-200 genes positively correlated (Pearson correlation) with the expression of either \textit{IL-6} or \textit{PDGFRA}, defining \textit{IL-6} and \textit{PDGFRA} signatures. When applied to our resistant model, the two signatures were enriched among the transcripts more expressed in the resistant than in the parental VL51 cells (Fig S17E), highlighting the similarities between our model and the clinical setting.

Finally, secreted levels of IL-6 were evaluated in the serum of patients treated with idelalisib, comparing CLL patients with primary or acquired resistance to the PI3Kδ inhibitor to patients responding to the drug and paired for similar clinical features (Table S6). In agreement with the \textit{in vitro} data, all samples but one secreting IL-6 were non-responders to idelalisib (Fig 8A). Longitudinal analyses, comparing responders and non-responders to idelalisib was carried out for those patients with IL-6 secretion and available data along the time. Non-responders showed increasing IL-6 levels upon treatment, while the paired responders remained with no IL-6 expression (Fig 8B). These data suggest that the secreted factors identified \textit{in vitro} can be present in the tumor microenvironment and that lymphoma cells might express the corresponding receptors to take advantage of available chemokines. Thus, albeit different mechanisms may drive resistance to PI3K inhibitors in clinical cases, the mechanism identified here might apply to some of these patients.

**Discussion**

We developed and characterized a model of secondary resistance to the PI3K inhibitors in splenic MZL. Our results indicate that: i) epigenetic reprogramming can drive resistance to PI3K inhibitors by promoting the secretion of cytokines; ii) the resistance can be overcome using various drugs, which could be tested in novel clinical trials.

Idelalisib was the first-in-class PI3Kδ inhibitor and several additional PI3K inhibitors with different selectivity, including duvelisib and copanlisib or umbralisib, have entered the clinical setting as single agents and in combinations (1-3). The model of secondary resistance we developed via prolonged \textit{in vitro} exposure to idelalisib presented decreased sensitivity to other PI3K inhibitors as copanlisib, duvelisib and umbralisib. Also, the role of the identified factors in the resistance to PI3K inhibitors has been further validated in additional B-cell lymphoma \textit{in vitro} models and across a large series of cell lines derived from different lymphoma types, and their expression demonstrated in various clinical specimens, including in the serum of idelalisib resistant patients.

Targeting epigenetic vulnerabilities has been recently proposed as a valuable strategy to overcome resistance to therapy in cancer, including PI3K inhibitors (30-33). Epigenetic cell plasticity, which might be pharmacologically reverted, allows the rise of drug tolerant subpopulations even in the absence of genetic lesions or can provide a first permissible environment for the emergence, later on, of cells carrying DNA changes (34). A relevant role for methylation has been suggested in splenic MZL, where hypermethylation phenotype, elevated expression of EZH2, and enrichment of PRC2-complex targets are associated with a more aggressive clinical outcome (16).
The resistant cell line exhibited secretion of interleukins and chemokines, such as IL-6, and up-regulation of pro-survival networks, including PDGFRA, JAK-STAT and NF-κB pathways. Our findings are in line with the notion that secreted factors can give resistance to PI3K inhibitors (35, 36), and with the background of the cell line we have used, representative of the recently described NNK splenic MZL subgroup, driven by mutations in genes involved in NF-κB /NOTCH/KLF2 (37). IL-6 can protect cancer cells from apoptosis and DNA-damage induced by drugs and can decrease the sensitivity to tyrosine kinase inhibitors by activating different signaling pathways, such as JAK-STAT, AKT-mTOR and NF-κB signaling pathways (38). Release of IL-6 mediates resistance to the BTK inhibitor ibrutinib in Waldenström’s macroglobulinemia (39), to duvelisib and copanlisib in DLBCL and T-cell lymphoma cells (35), and, in head and neck squamous cell carcinoma cell lines, blockade of IL-6 signaling overcomes resistance to the pan PI3K inhibitor buparlisib (36). In our resistant cell line, the up-regulation of IL-6 was associated with the downregulation of let-7 and miR-125 microRNAs, which directly target the interleukin (19), and with the loss of methylation in the IL-6 gene promoter. The latter was indeed methylated in the parental cell line but not in resistant lines, and methylation-based repression of the IL-6-targeting miRNAs, miR-125 and members of let-7 family, might contribute to the increased IL-6 expression. LIN28 is an RNA binding protein that functions as oncogene inhibiting the expression of let-7 family microRNAs, dysregulating the normal balance between differentiation and cell growth (40). Since IL-6 induces transcription of NF-κB targets, a positive feedback loop leads to B-cell activation under the control of LIN28 and let-7 (19). The miRNA mir-125 is located at 19q13 and frequently downregulated or deleted in cancer, and it can also regulate cell proliferation by targeting p53 (41). Loss of miR-125 leads to high expression of STAT3, IL-6 itself and of a subunit of IL-6 receptor complex (IL-6R) (20). Hence, in the context of NF-κB and JAK-STAT activation, miR-125 might work concomitantly with let-7. Alongside with IL-6, also PDGFRA was upregulated in the resistant cell line. Active PDGFRA signaling, as a consequence of genomic aberrations affecting PDGFRA, is associated with tumor development and progression in solid cancer (42), it mediates the activation of PI3K-AKT, JAK-STAT and RAS-ERK signaling (43), and it confers resistance to the tyrosine kinase inhibitor imatinib (44). PDGFR signaling is known to increase IL-6/IL-6R axis expression (45), and, furthermore, PDGFR is also linked with CXCR4 (46), upregulated in the resistant cells. CXCR4 is upregulated after exposure to BCR inhibitors, including PI3K and BTK inhibitors (26) and, in DLBCL models, it has been suggested as a potential mechanism of resistance to the tyrosine kinase inhibitors themselves (5, 6).

Since a multitude of results, including genetic silencing and use of IL-6 recombinant protein, indicated that the mechanism of resistance to PI3K inhibitors in the VL51 model was driven by the activation of IL-6/STAT3 and PDFGRA signaling cascades and dependent on the action of LIN28, we explored possible therapeutic interventions. Tocilizumab is an IL-6R blocking antibody in clinical use to treat different autoimmune disorders and more recently for CRS that can be seen with chimeric antigen receptor (CAR)-T cell therapy or in patients with coronavirus disease 2019 (COVID-19) (24). Masitinib, a small molecule that inhibits PDGFR signaling and targets the innate immune system, is under clinical development for various indications, including systemic mastocytosis, solid tumors, and amyotrophic lateral sclerosis (47). The combination with these two already clinically available drugs, and with the LIN28 inhibitor LIN1632 or with the STAT3 inhibitor statin, two preclinical compounds, restored sensitivity to PI3K inhibition. Moreover, the resistant cells had higher CD19 expression on their cell surface, which gave a much higher activity to the CD19 targeting antibody drug conjugate loncastuximab tesirine, recently FDA approved for the treatment of patients with relapsed or refractory large B-cell lymphoma (25). In the clinical routine, CD19 could be easily investigated (by immunohistochemistry or flow cytometry) and provide rationale for testing CD19-targeting antibody drug conjugates, naked antibodies, and cellular therapies in patients exposed to PI3K inhibitors.

In conclusion, a model of secondary resistance to the PI3K inhibitors, derived from splenic MZL, have revealed mechanisms of resistance to the drug and allowed the identification of a first series of active therapeutic approaches that can be further explored.
References


Figure 1. Profiles of drug sensitivity differ between parental and resistant lines. Acquired resistance was tested by MTT assay (72h) in parental (black) and resistant (red) cells of VL51 line. Drug sensitivity was evaluated in resistant and parental cells for the PI3K inhibitors idelalisib (A), copanlisib (B), duvelisib (C), umbralisib (D), the dual PI3K-mTOR inhibitor bimiralisib (E), the mTOR inhibitor everolimus (F) and the chemotherapy agent and MDR substrate vincristine (G). Error bars correspond to standard deviation of the mean; µM for micro molar. Data derived from at least three independent experiments. P values from a Z-test, statistically significant for P<0.05.

Figure 2. Multi-omics signature of VL51 resistant identifies activation of IL-6-PDGFRA axis. Heatmap of RNA (GEP), methylation and miRNA profiles of resistant compared to parental. Heatmap values represent the differences between resistant and parental: fold change (log2 for RNA and miRNA) or delta Beta-value (methylation), red for enrichment in resistant and blue for parental. Columns correspond to gene expression (GEP, RNA-seq) and methylation (MethylationEPIC BeadChip, Illumina) profiles of the top-10 up-regulated and top-10 down-regulated genes; rows represent the differently expressed miRNAs (RNA-seq) with values in the column corresponding to the targeted gene. * for statistically significant differences (moderated t-test).

Figure 3. Proteome profiles of resistant differ from parental. (A) Cell viability by MTT assay of parental cells (black line) exposed to idelalisib in presence or not of conditioned medium from VL51 idelalisib-resistant (red line), cultured for 48h, Parental + RES-cond RPMI, dotted grey line). Data derived from the average of three independent experiments. µM for micro molar. (C) Expression of surface PDGFRA (top), CXCR4 (center) and CD19 (bottom) by FACS in parental (grey) and resistant (red) lines. Dotted black line for negative control (neg-cnt). Data derived from two independent experiments. (D) Levels of protein phosphorylation by immunoblot in resistant cells. Values correspond to average fold-change of resistant compared to parental in two independent experiments. Data was normalized to GAPDH levels. Error bars represent standard error of the mean. * for statistically significant differences (t-test).

Figure 4. Interfering with IL-6 or PDFGRA overcomes resistance in VL51 model. (A) Small interfering RNAs were used for gene expression silencing of IL-6 alone (brown dotted line), PDGFRA alone (orange dotted line) or concomitant silencing of IL-6 and PDGFRA (yellow dotted line). Black and red lines for parental and resistant controls. * for statistically significant differences when compared to resistant control (red line, Z-test P<0.05). (B) Stimulation with recombinant IL-6 (30ng/mL) conferred resistance to idelalisib in the parental cells (dotted black line) and blocking of IL-6 signaling with the monoclonal antibody tocilizumab (25µg/mL) overcome resistant to idelalisib (red dotted line). Black and red continuous lines for parental and resistant controls. * for statistically significant differences when compared to parental control (black continuous line, Z-test P<0.05). Sensitivity to all treatments was tested by MTT assay upon 72h. Data derived from three independent experiments.

Figure 5. Combination with LIN28 or PDFGRA inhibitors recovers sensitivity to idelalisib. (A) Addition of 1µM of the LIN28 inhibitor LIN1632 recover sensitivity to idelalisib in the resistant with no effect in the parental and very limited sensitivity as a single agent for both parental and resistant lines (Fig S11B). (B) Combination of idelalisib with 500nM of the PDGFRA inhibitor masitinib increases sensitivity to idelalisib in the resistant with limited benefit in the parental. Single treatment of masitinib was beneficial only in resistant but not in parental (Fig S13B). Sensitivity to all treatments was tested by MTT assay upon 72h. Data derived from three independent experiments, error bars represent standard deviation of the mean. * for statistically significant differences when compared to parental control (black continuous line, Z-test P<0.05).

Figure 6. Idelalisib-resistant cells exhibited increased sensitivity to an anti-CD19 treatment. CD19 expression was increased in resistant (red) compared to parental (black) cells, either at mRNA (RNA-seq, A) or surface (FACS, Fig 3B bottom panel) levels. Response to loncastuximab tesirine (ADCT-402) was tested by MTT assay upon 72h of exposure (B). Sensitivity to all treatments was tested by MTT assay upon 72h. Data derived from three independent experiments, error bars represent standard deviation of the mean. * for statistically significant differences between parental (black) and resistant (red) (Z-test, P<0.05).

Figure 7. Expression levels of crucial factors are correlated with resistance to idelalisib. IL-6, PDGFRA, LIN28 expression levels are inversely correlated with idelalisib sensitivity. Conversely expression of miR-125a and members of let-7 family of microRNAs is associated with sensitivity to idelalisib. Expression and sensitivity data were analyzed from a previous publication of our group in a panel of 34 B-cell lymphoma cell lines (26). Cell lines were split in two groups based on higher or lower values than the median expression of the corresponding gene or miRNA. Mean of idelalisib IC50 were calculated for these two groups and compared by t-test. * for P<0.05.
Figure 8. Higher IL-6 levels is detected in serum from idelalisib resistant than sensitive CLL patients. (A) IL-6 secretion was evaluated in the serum of CLL samples at the end of the treatment by ELISA (Luminex, R&D Systems). Patients responding (responders, blue) or non-responding (non-responders, red) to idelalisib were compared by t-test. P for adjusted p-value. (B) Longitudinal analyses were performed on those clinically-paired patients with secretion of IL-6. Secretion of IL-6 was compared between non-responding to idelalisib (red) and their corresponding matched controls (responding, blue). MFI: mean fluorescence intensity.
Figure 1

(A) Idelalisib

(B) Copanlisib

(C) Duvelisib

(D) Umbralisib

(E) Bimiralisib

(F) Everolimus

(G) Vincristine

Legend

- Black: Parental
- Red: Resistant

% Viability vs. Concentration (μM)

P-values: P < 0.01, P > 0.05
Figure 2
Figure 3

Panel A: Graph showing the percentage viability of cells treated with Idelalisib at different concentrations. The graph compares Resistant, Parental, and Parental + RES-cond RPMI cell lines. The data is represented with error bars, and the significance level is P < 0.01.

Panel B: Density plots for PDGFRA, CXCR4, and CD19 showing the distribution of different cell lines in relation to the Y-axis (Density) and the X-axis (Log10 MFI (FACS)). Each plot indicates a significant difference at P < 0.01.

Panel C: Bar chart showing the protein quantification (fold change resistant vs parental) for pAKT, pERK, pJAK2, pSTAT3 S727, and pSTAT3 Y705. The chart includes error bars, and the significance level is indicated by an asterisk (*) where applicable.

Legend:
- pAKT
- pERK
- pJAK2
- pSTAT3 S727
- pSTAT3 Y705
Figure 6

A

CD19 (RNA-seq)

CD19 expression (RNA-seq)

parental | resistant

B

ADCT-402

% Viability

Concentration (pM)

Parental | Resistant

*
Figure 8

A  
Idelalisib treated samples

P = 0.069

Legend

- red: non-responders
- blue: responders

B  
FB3-FB4  
FB15-FB16  
FB17-FB18

Days on treatment

IL6. MFI by ELISA

Days on treatment

IL6. MFI by ELISA

Days on treatment

IL6. MFI by ELISA