ARTICLE

Identification of PIKfyve kinase as a target in multiple myeloma

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ABSTRACT

The cellular cytotoxicity of APY0201, a PIKfyve inhibitor, against multiple myeloma was initially identified in an unbiased *in vitro* chemical library screen. The activity of APY0201 was confirmed in all 25 cell lines tested and in 40% of 100 *ex vivo* patient-derived primary samples, with increased activity in primary samples harboring trisomies and lacking t(11;14). The broad anti-multiple myeloma activity of PIKfyve inhibitors was further demonstrated in confirmatory screens and showed the superior potency of APY0201 when compared to the PIKfyve inhibitors YM201636 and apilimod, with a mid-point half maximal effective concentration (EC_{50}) at nanomolar concentrations in, respectively, 65%, 40%, and 5% of the tested cell lines. Upregulation of genes in the lysosomal pathway and increased cellular vacuolization were observed *in vitro* following APY0201 treatment, although these cellular effects did not correlate well with responsiveness. We confirm that PIKfyve inhibition is associated with activation of the transcription factor EB, a master regulator of lysosomal biogenesis and autophagy. Furthermore, we established an assay measuring autophagy as a predictive marker of APY0201 sensitivity. Overall, these findings indicate promising activity of PIKfyve inhibitors secondary to disruption of autophagy in multiple myeloma and suggest a strategy to enrich for likely responders.

Introduction

Although the survival outcomes of patients with multiple myeloma (MM) have improved significantly, in the majority of patients the disease remains characterized by recurrent episodes of relapse. Identification of vulnerable targets, particularly those targeting plasma cell biology, is thus an attractive approach aiming towards advances of therapeutic strategies. Consequently, we utilized an *ex vivo* chemo-genomics screening approach to identify potentially unrecognized targets in this disease. As part of this study, and somewhat unexpectedly, PIKfyve was identified as a vulnerable target in MM.

PIKfyve, first described in 1999,[1] is a mammalian protein and lipid kinase that controls complex and distinct cellular functions (reviewed by Shisheva *et al.*[2]). PIKfyve phosphorylation of protein substrates includes autophosphorylation[3] while lipid kinase activity generates two phosphorylated species of phosphatidylinositol (PtdIns):-5-P and -3,5-P2.[4] PtdIns-5-P plays crucial roles in remodeling the actin cytoskeleton, endocytosis, and nuclear signaling, and is suggested to be a critical signal in innate immune responses. PtdIns-3,5-P2 is involved mainly in intracellular trafficking and lysosomal acidification and is thus crucial for the regulation of macroautophagy (herein denominated autophagy).[5] Apilimod, initially identified in functional screens as negatively interfering with the production of interleukin (IL-)12 and IL-23[6] and used in clinical trials of the treatment of inflammatory diseases[7-10] was subsequently characterized as a PIKfyve inhibitor.[11] Interestingly, apilimod inhibition has recently been described as a potentially useful therapeutic approach for the treatment of non-Hodgkin lymphoma (NHL).[12]

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Other novel compounds targeting the PIKfyve kinase, APY0201, YM201636, AS2677131, AS2795440, and MF4, have followed.[13-16] Here we report that APY0201 and other PIKfyve inhibitors are effective inhibitors of MM cell viability in *in vitro* and *ex vivo* models of MM, explore their mechanisms of action, and describe the development of a predictive assay for PIKfyve sensitivity.

Methods

PIKfyve inhibitor sensitivity

APY0201 was included in a 76-drug panel high throughput screen and evaluated in a 7-point, 10-fold dilution of drug concentration, starting at 10 μM. Twenty-five human MM cell lines (HMCL) and 15 NHL cell lines were incubated for 24 or 72 h. Cellular viability was assessed with the CellTiter Glo (Promega) assay for all dose-response curves. Mid-point half maximal effective concentrations (herein denominated EC_{50}), maximum inhibition, and area under the curve (AUC) were calculated.[17]

Twenty HMCL were treated with a 20-point 2-fold dilution of drug concentration, starting at 40 μM, and incubated for 72 h with APY0201 (MedChemExpress, HY-15982, Monmouth Junction, NJ, USA), apilimod (Santa Cruz Biotechnology, sc-480051, Dallas, TX, USA), and YM201636 (SelleckChem, S1219, Houston, TX, USA).

Ex vivo sensitivity to APY0201 was assessed after 24 h incubation in 100 purified patient-derived MM samples (through magnetic bead sorting for $CD138^+$ cells; average purity greater than 95%). Fifteen samples were also screened against APY0201 and apilimod in a 14-point, 3-fold dilution of drug concentration, starting at 50 μM, and incubated for 72 h. Leukocytes from whole bone marrow samples were incubated for 24 h with increasing concentrations of APY0201 to measure cytotoxicity, as described previously.[18] Written informed consent was obtained from the patients and samples were collected and stored under Mayo Clinic Institutional Review Board approval (IRB 919-04, 2207-02, 15-009436, and 18-003198). This study was conducted in accordance with the Declaration of Helsinki.

Immunoblotting

Anti-β-actin (#A00702-100) antibody was purchased from GeneScript (Piscataway, NJ, USA), anti-Lamp-1 (#ab25630) was purchased from Abcam (Cambridge, MA, USA), anti-SQSTM1 (#sc-28359) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA), and anti-cathepsin A (#AF1049) and anti-cathepsin D (#AF1014) were purchased from R&D Systems (Minneapolis, MN, USA). Antibodies against β-tubulin (#2128), Beclin1 (#3495), Caspase 3 (#9662), GAPDH (#2118), Lamin A/C (#4777), LC3A/B (#12741), PARP (#9542), and transcription factor EB (TFEB, #4240) were purchased from Cell Signaling Technology (Danvers, MA, USA).

Autophagy organelle formation

Vacuolar phenotype was evaluated by live cell differential interference contrast (DIC) imaging. Acidic vacuoles were identified with the LysoSensor Yellow/Blue DND-160 probe (#L7545, Thermo Fisher Scientific, Waltham, MA, USA). HMCL were incubated with 20 μL of the Premo Autophagy Tandem Sensor RFP-GFP-LC3 kit (#P36239, Thermo Fisher Scientific) for 48 h with subsequent addition of dimethylsulfoxide (DMSO) control or APY0201 for 18 h. The Autophagy Detection Kit (Abcam #ab139484) was used according to the manufacturers' recommendations. Cellular viability was measured at 72 h.

mRNA sequencing

HMCL were treated with 100 nM and patients' samples with 50 and 500 nM of APY0201, and DMSO as a control, for 24 h; the cells were then harvested, and total RNA was extracted. An internally developed RNA-sequencing analysis workflow (MAPRSeq,[19] v3.0.1) was used to perform comprehensive analysis of raw RNA sequencing paired-end reads, which were aligned by a fast and splice-aware aligner (STAR,[20] v 2.5.2b) to human genome build hg38. Quality control was performed with RSeQC (v3.0.0).[21] Gene expression was quantified with featureCounts[22] from the Subread package *(http://subread.sourceforge.net/*, v1.5.1). Genes were determined as differentially expressed based on log-fold change >1, and the Enrichr platform[23] was used to identify involved pathways. The data have been deposited in the National Center for Biotechnology Information's (NCBI) Gene Expression Omnibus (GEO),[24] accessible through GEO series accession number GSE134598.

Results

Identification of PIKfyve as a potential target in multiple myeloma and non-Hodgkin lymphoma

Medium throughput screening on MM and NHL cell lines was first conducted at Nanosyn Inc. (Santa Clara, CA, USA) using a panel of known Food and drug Administration-approved oncology drugs, kinase inhibitors, and epigenetic compounds assembled by the investigators. A more refined panel of 76 drugs was then selected and sensitivity to the single agents was evaluated in 25 MM and 15 NHL cell lines. We observed a dose dependent inhibition of cellular viability in all of the 25 HMCL exposed to the PIKfyve inhibitor APY0201 after 72 h incubation, with a median EC_{50} of 55 nM and a median maximum inhibition of cellular viability of 81% (dose-response curves are shown in *Online Supplementary Figure S1*). These results prompted a confirmatory validation screen of 20 HMCL treated with APY0201 and two additional PIKfyve inhibitors, YM201636 and apilimod, with an increased dose range. Dose-dependent inhibition of cellular viability was observed in this confirmatory screen in 20 HMCL with all three PIKfyve inhibitors after 72 h incubation. APY0201 was the most potent PIKfyve inhibitor, followed by YM201636 and apilimod (Table 1, *Online Supplementary Figures S2-S4* and *Online Supplementary Table S1*).

In NHL cell lines the effect was similar; after 72 h incubation, APY0201 was the most potent PIKfyve inhibitor evaluated, with 14 of 15 tested lines (93%) having an EC_{50} in the nanomolar range (median 76 nM). For YM201636, only 37.5% of eight tested NHL cell lines were sensitive at nanomolar concentrations with a median EC_{50} of 530 nM. Finally, for apilimod, only one of eight tested NHL lines (12.5%) had nanomolar activity with an EC_{50} of 405 nM (*Online Supplementary Figures S5* and *S6* and *Online Supplementary Table S2*).

Ex vivo anti-myeloma activity of multiple PIKfyve inhibitors

We next tested 100 *ex vivo* primary $CD138^+$-selected patients' samples. Dose-dependent sensitivities for APY0201 were observed in 40% of these *ex vivo* samples at 24 h, with 47% of the responsive samples exhibiting an EC_{50} lower than 100 nM (19% of all tested samples). The

sensitivity to both APY0201 and apilimod was then examined in 15 *ex vivo* primary patients' samples after 72 h incubation, and more significant anti-MM activity was noted. Over 90% of the primary patients' samples showed dose-dependent inhibition of cellular viability in response to both drugs. APY0201 was considered the more potent PIKfyve inhibitor, with 71% of the samples exhibiting sensitivity in the nanomolar range (median EC_{50} 179 nM), while for apilimod, 93% of the samples exhibited sensitivity in the micromolar range (median EC_{50} 22618 nM) (Table 2 and *Online Supplementary Table S3*).

The cytotoxicity of APY0201 for different leukocyte populations was evaluated in two primary patients' samples with increasing drug concentrations. The plasma cell population of the first sample was sensitive to APY0201, with a marked decrease in cellular viability when incubated with the highest dose of the drug (*Online Supplementary Figure S7A*); the second sample was considered resistant (*Online Supplementary Figure S7B*). We did not observe any significant off-target effect of APY0201 in the T and B lymphocyte populations in either sample, while some off-target effects were noted in the population composed of monocytes, macrophages, and granulocytes.

Ploidy and t(11;14) are biomarkers of sensitivity

In HMCL, APY0201 was active regardless of the presence or absence of IgH translocations, *TP53* deletion, *CKS1B* gain, monosomy 13, and *MYC* rearrangements (*Online Supplementary Figure S8*). We then evaluated possible associations between APY0201 sensitivity in primary patients' samples and clinical findings. We examined different disease stages (monoclonal gammopathy of undetermined significance, smoldering MM, and MM), disease status (newly diagnosed or relapsed), fluorescence *in situ* hybridization cytogenetics [t(11;14), t(4;14), t(14;16), t(6;14), deletion of 17p, *CSK1B* duplication, *MYC* rearrangement, monosomy 13, and trisomies of one or more odd-numbered chromosomes], flow cytometry analysis (hyperdiploid status and S-phase), and hyperdiploidy according to Wuilleme *et al.*[25] Despite the limited sample size, primary patients' samples lacking trisomies (Fisher test, P=0.0232) and harboring t(11;14) (Fisher test, P=0.0189) were more frequently classified as inactive to APY0201 in the *ex vivo* drug screen (Figure 1A, B). With high-risk patients defined as those with t(4;14), t(14;16), deletion of 17p, or *CSK1B* duplication, no discernible difference in PIKfyve sensitivity was observed between patients with high- or low-risk genetics.

Data publicly reported from the CoMMpass project (these data were generated as part of the Multiple Myeloma Research Foundation Personalized Medicine Initiatives; *https://research.themmrf.org and www.themmrf.org*) showed lower *PIKFYVE* expression levels in hyperdiploid samples when compared to non-hyperdiploid samples (t-test, P=0.0003) and in samples lacking t(11;14) when compared to samples harboring the translocation (t-test, P<0.0001). These findings were also confirmed in a cohort of 487 primary MM samples (Mann-Whitney test, P<0.0001) (*unpublished data*). We hypothesize that increased *PIKFYVE* expression levels may be associated with increased resistance to PIKfyve inhibitors, with higher concentrations of the inhibitors necessary to activate TFEB. However, further studies are needed to support this hypothesis.

Upregulation of lysosomal pathways *in vitro* and *ex vivo*

To further elucidate the anti-MM mechanism of PIKfyve inhibitors, we evaluated the mRNA-sequencing profiles of five HMCL with low, intermediate and high sensitivities to APY0201. When comparing RNA expression profiles from each HMCL treated with APY0201 for 24 h with its untreated control using the Enrichr platform, gene targets of the lysosomal pathway were upregulated in all five HMCL (P≤0.01), corroborating previous findings.[26] This is consistent with previous data from B-NHL suggesting that apilimod impaired lysosomal function and consequent degradation of the autophagosomal cargo.[12]

Three primary patients' samples identified as sensitive to APY0201 (EC_{50} of 56.29, 101.14, and 103.36) and one resistant sample, lacking a dose-response curve (*Online Supplementary Figure S9*), were sent for mRNA-sequencing analysis. The three sensitive patients' samples harbored

Table 1. Cellular viability inhibition of 20 human myeloma cell lines after 72 h of incubation with three PIKfyve inhibitors.

Mid-point EC_{50}	HMCL					
	APY0201		YM201636		Apilimod	
	Samples	%	Samples	%	Samples	%
<100 nM	5/20	25	0/20	0	0/20	0
100-1000 nM	8/20	40	8/20	40	1/20	5
>1000 nM	7/20	35	12/20	60	19/20	95

EC_{50}: half maximal effective conentration; HMCL: human myeloma cell lines.

Table 2. Cellular viability inhibition of *ex vivo* primary patients' samples after 24 and 72 h incubation with APY0201 and apilimod.

Mid-point EC_{50}	*Ex vivo* Primary Patients' Samples					
	APY0201 24 Hour Incubation		APY0201 72 Hour Incubation		Apilimod 72 Hour Incubation	
	Samples	%	Samples	%	Samples	%
<10 nM	2/100	2	3/14	21.4	0/15	0.0
10-100 nM	17/100	17.0	2/14	14.3	0/15	0.0
100-1000 nM	11/100	11.0	5/14	35.7	1/15	6.7
>1000 nM	10/100	10.0	3/14	21.4	14/15	93.3
Inactive	60/100	60.0	1/14	7.2	0/15	0.0

EC_{50}: half maximal effective conentration.

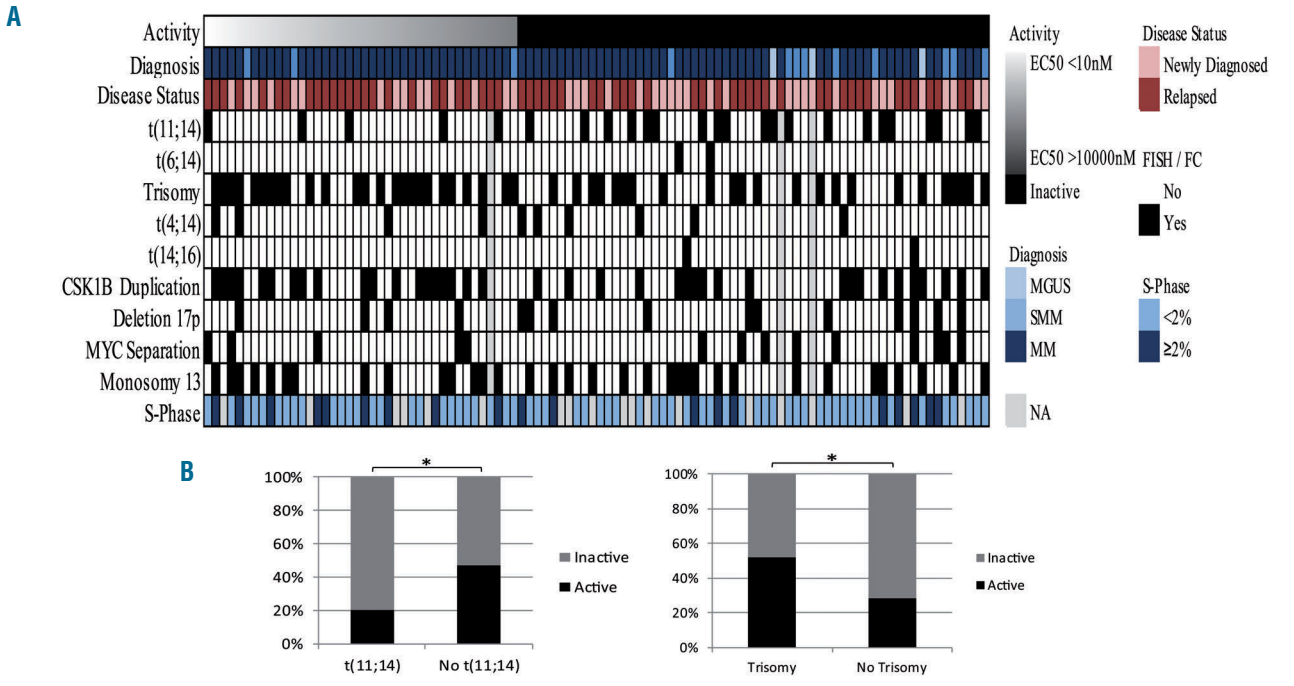

Figure 1. A global overview of clinical data from 100 multiple myeloma patients with *ex vivo* samples tested for APY0201 activity. (A) Clinical data including diagnosis (monoclonal gammopathy of undetermined significance, smoldering multiple myeloma and multiple myeloma), disease status (newly diagnosed or relapsed), fluorescence *in situ* hybridization data (t(11;14), t(6;14), trisomies of one or more odd-numbered chromosomes, t(4;14), t(14;16), *CSK1B* duplication, deletion of 17p, *MYC* rearrangements, and monosomy 13), and S-phase status from flow cytometry analysis. Missing data are shown in gray. (B) Primary patients' samples with trisomies of one or more odd-numbered chromosomes (P=0.0182) and lack of t(11;14) (P=0.0168) had increased *ex vivo* sensitivity to APY0201. EC_{50}: half maximal effective concentration; FISH: fluorescence *in situ* hybridization; FC: flow cytometry; MGUS: monoclonal gammopathy of undetermined significance; SMM: smoldering multiple myeloma; MM: multiple myeloma

trisomies of one or more odd-numbered chromosomes, further supporting an increased sensitivity of hyperdiploid MM to APY0201. As demonstrated in HMCL, incubation with APY0201 at 500 nM for 24 h led to upregulation of the lysosomal pathway in both sensitive and resistant primary patients' samples ($P \leq 0.01$). The transcriptional upregulation of lysosomal biogenesis in HMCL and in primary patients' samples warranted further assays to evaluate the functional consequences of PIKfyve inhibition on lysosomes and autophagy.

Of interest *CCL3* was the single most upregulated gene across all five HMCL and all four primary patients' samples following APY0201 treatment for 24 h. MM cells constitutively secrete *CCL3* (also known as MIP-1α) and the chemokine has been directly related to osteolytic bone lesions and poor prognosis by stimulating the proliferation, migration and survival of MM cells (reviewed by Aggarwal *et al.*[27]). In MM harboring t(4;14), we have previously demonstrated that CCL3 is downregulated by inhibition of *FGFR3* and is regulated by the RAS-MAPK pathway, with overactivation of RAS-MAPK upregulating *CCL3*.[28] This counter-intuitive link between PIKfyve inhibition, lysosomal pathways and *CCL3* upregulation deserves further exploration beyond the scope of this report.

APY0201 activated TFEB, a master regulator of lysosomal biogenesis and autophagy, and promoted cellular vacuolization

Higher basal protein levels of TFEB, a regulator of lysosomal function and autophagy,[29] was shown in two HMCL (KMS26 and JJN3) most sensitive to APY0201 when compared to the two most resistant HMCL (RPMI-8226 and EJM), corroborating previous data associating TFEB overexpression with sensitivity to apilimod in B-cell NHL (Figure 2A, B).[12] Following treatment with APY0201 for 6 h, activation of TFEB, found in its dephosphorylated state, was observed independently of the HMCL sensitivity profile to the compound. Nuclear translocation of TFEB following PIKfyve exposure was subsequently confirmed in a subcellular localization immunoblotting assay (Figure 2C, D). Dephosphorylated TFEB translocates from the cytoplasm to the nucleus to regulate the expression of target genes associated with autophagy.[30] Therefore, these findings further supported an APY0201-induced autophagy disturbance through PIKfyve inhibition in MM.

Treatment with APY0201 led to the formation of enlarged intracellular vacuoles in all three HMCL (KMS26, L363, and EJM), independently of drug sensitivity (Figure 2E). The endolysosomal swelling phenotype has been widely related to the disruption of PIKfyve activity[11–13,15,16,26,31] due to the role of the kinase in vacuole maturation after lysosome fusion has occurred.[31] Vacuolation could therefore be a phenotypic biomarker of PIKfyve inhibition and consequent reduction of PtdIns-3,5-P2.[14] The acidic nature of the vacuoles was further examined with LysoSensor staining. Blue fluorescence found in more neutral environments was observed in the vacuoles of all four treated HMCL (2 sensitive and 2 resistant to APY0201), indicating the prevalence of less acidic vacuolar content and, therefore, suggesting impaired lysosomal function. However, yellow fluores-

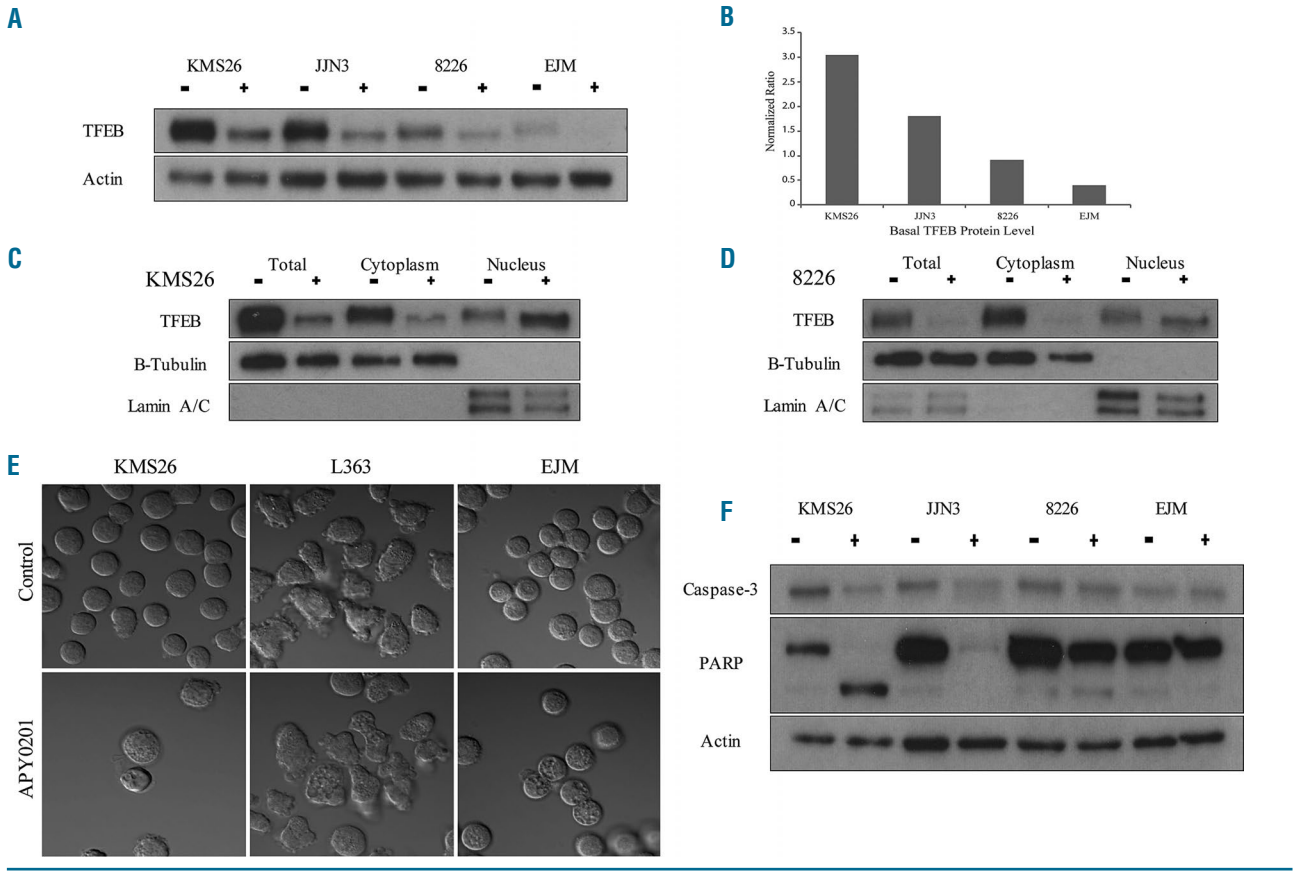

Figure 2. APY0201 treatment leads to activation of TFEB, a vacuolar phenotype, and cell death by apoptosis in human myeloma cell lines. (A) Two sensitive (KMS26 and JJN3) and two resistant (RPMI-8226 and EJM) human myeloma cell lines (HMCL) were treated with dimethylsulfoxide (DMSO) or 100 nM of APY0201. Cell lysates were harvested after 6 h of incubation with the drug and immunoblotting was performed to determine the level of transcription factor EB (TFEB) protein. (B) Basal TFEB level was correlated to sensitivity. Two HMCL were treated with DMSO or 100 nM of APY0201 after 6 h for KMS26 (C) and 24 h for RPMI-8226 (D) with immunoblotting performed to determine the subcellular localization of TFEB. (E) Three HMCL were or were not treated with APY0201 at 100 nM and live cell differential interference contrast (DIC) imaging was performed after 48 h with a confocal microscope Zeiss LSM 800 (63X). (F) Four HMCL were treated with DMSO (-) or 100 nM of APY0201 (+) for 48 h prior to immunoblotting for caspase-3 and poly (ADP-ribose) polymerase (PARP) protein.

cence found in more acidic environments prevailed in the vacuoles of the two resistant HMCL, RPMI-8226 and EJM, indicating a partial maintenance of a more acidic content after APY0201 exposure, which is necessary for autophagic flux maintenance (*Online Supplementary Figure S10*). The galectin puncta assay was performed to determine whether lysosomal cell death was critical in the APY0201-induced cytotoxicity. Lysosomal cell death is promoted by leakage of lysosomal contents following permeabilization of the lysosomal membrane;[32] however, no evidence of lysosomal membrane permeabilization was observed (*data not shown*). APY0201 apoptosis-mediated cellular toxicity was demonstrated by immunoblotting of Caspase-3 and PARP cleavage in the sensitive HMCL, KMS26 and JJN3, but not in the two resistant HMCL, RPMI-8226 and EJM (Figure 2F).

Multiple myeloma cells resistant to PIKfyve inhibitors partially maintain autophagic flux

To further evaluate autophagic flux, we examined key components of lysosomes and autophagosomes. An accumulation of the inactive precursor procathepsin D was observed in all four HMCL and of procathepsin A both in sensitive KMS26, JJN3, and in resistant EJM cells following incubation with APY0201 (Figure 3A), demonstrating a decrease in maturation of lysosomal proteases independent of APY0201 sensitivity. All treated HMCL exhibited an increase in LC3 A/B-II, a protein present in autophagosomes and autophagolysosomes; as well as lysosome-associated membrane protein 1 (Lamp-1), a protein present in lysosomes and autophagolysosomes. An increase in sequestosome 1 (SQSTM1)/p62 protein levels was also demonstrated in all four HMCL, further highlighting impaired autophagic protein degradation (Figure 3B). We therefore confirmed that lysosomal function, and consequently autophagy, was disrupted following PIKfyve inhibition and did not seem to predict drug sensitivity.

However, Beclin-1, a promoter of autophagy was decreased in KMS26 and JJN3 (the two HMCL sensitive to PIKfyve inhibitors) and discretely increased in RPMI-8226 and EJM (the two PIKfyve inhibitor-resistant HMCL) (Figure 3B). The SQSTM1:Beclin1 protein level ratio was then used as an autophagy indicator, with decreased SQSTM1 and increased Beclin-1 indicative of increased autophagic flux, as described previously.[33] A higher ratio was found in the sensitive HMCL (KMS26 and JJN3) when compared to the resistant HMCL (Figure 3C), which demonstrates impaired autophagic flux in drug-sensitive

HMCL. Further evaluation with the LC3-fluorescent protein showed a reduction of the acid-sensitive green fluorescent protein and maintenance of the acid-insensitive red fluorescent protein in the representative resistant HMCL, EJM, when compared to the sensitive HMCL, JJN3, after 18 h of treatment with APY0201, indicating maturation of autophagosomes to autophagolysosomes in the resistant HMCL (Figure 3D, E).

Finally, we performed a cellular viability assay treating the two representative resistant HMCL, RPMI-8226 and EJM, with 1 nM and 2 nM of the autophagy inhibitor bafilomycin A1, respectively, showing synergistic anti-MM activity (Figure 3F, G). In summary, these findings demonstrate the key role of autophagic flux disruption in MM cytotoxicity caused by PIKfyve inhibitors (Figure 4). Resistant HMCL maintain functional autophagy more efficiently, representing a mechanism of resistance to these drugs.

APY0201 sensitivity *in vitro* and *ex vivo* was predicted with the autophagy detection kit

Since anti-MM activity of APY0201 was associated with disruption of lysosome function and autophagy, we explored an autophagy detection assay, as a biomarker to

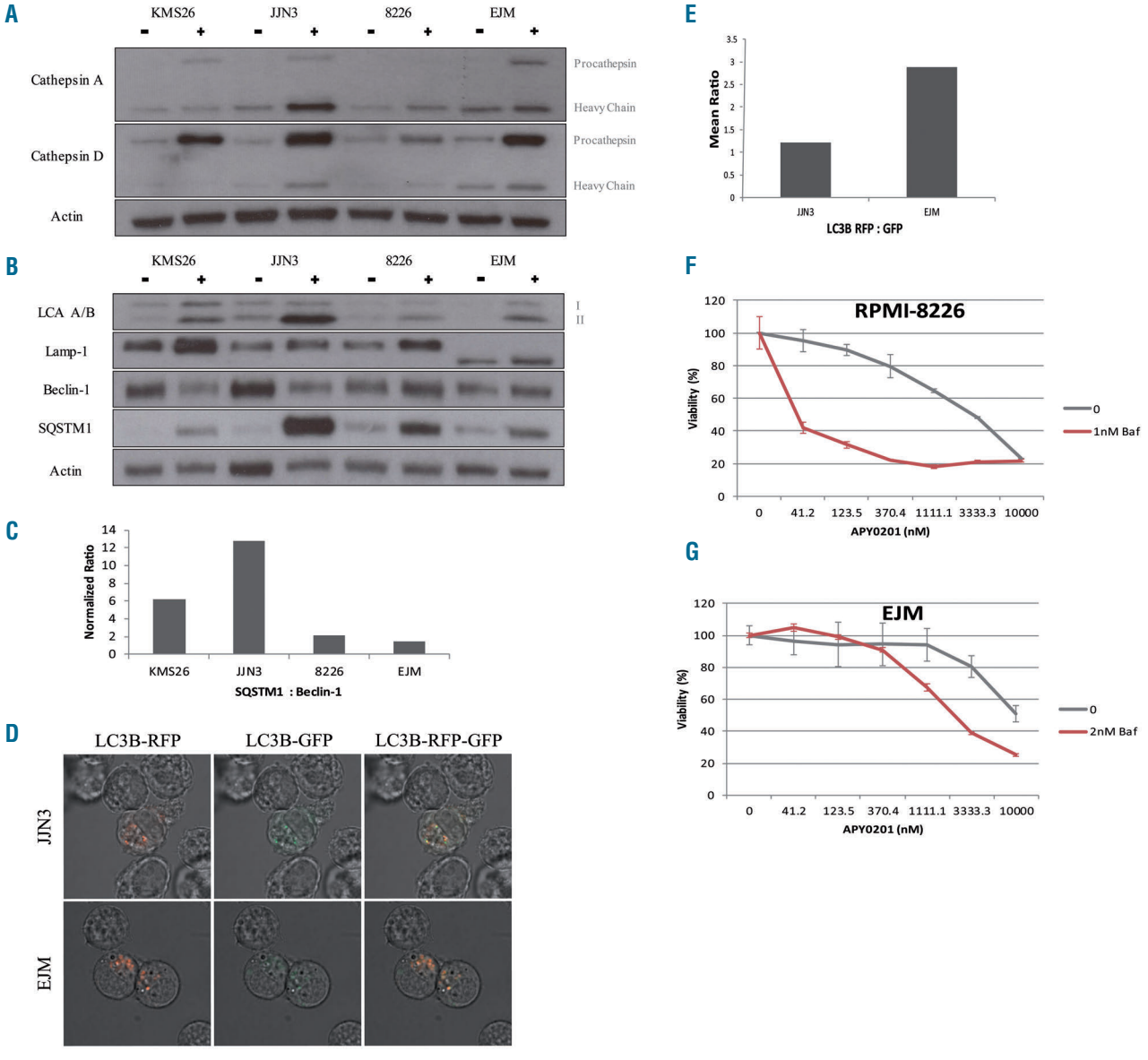

Figure 3. APY0201 treatment leads to an increase in inactive precursors of lysosomal proteases and in autophagy organelles but decreased autophagic flux in sensitive human myeloma cell lines. Four human myeloma cell lines (HMCL) were treated with dimethylsulfoxide (DMSO) or 100 nM of APY0201. Cell lysates were harvested after 48 h of drug incubation and immunoblotting was performed to determine the levels of cathepsin A and D proteins (A) and Beclin-1, LC3 A/B, lysosome-associated membrane protein 1 (Lamp-1), and sequestosome 1 (SQSTM1)/p62 protein levels (B). (C) The ration of SQSTM1:Beclin1 protein levels in two sensitive (KMS26 and JJN3) and two resistant (RPMI-8226 and EJM) HMCL following 48 h of incubation with APY0201. (D) LC3 acid-sensitive green fluorescent protein (GFP) and -insensitive red fluorescent protein (RFP) in representative sensitive and resistant HMCL (JJN3 and EJM, respectively) after 18 h of treatment with 100 nM of APY0201; images were obtained with a Zeiss LSM 800 confocal microscope (63X) and processed with Zen Blue software. (E) Mean LC3 RFP:GFP ratio in JJN3 and EJM. (F) Treatment of the RPMI-8226 resistant HMCL with APY0201 with and without 1 nM of bafilomycin A1 after 48 h. (G) Treatment of the EJM-resistant HMCL with APY0201 with and without 2 nM of bafilomycin A1 after 48 h.

predict sensitivity to APY0201. A more pronounced autophagy signal occurred when three HMCL sensitive to APY0201 (KMS26, JJN3, and FR4) were treated with the PIKfyve inhibitor for 18 h than when three resistant HMCL (EJM, KMS28PE, RPMI-8226) were so treated (Figure 5A). A decrease in cellular viability was confirmed as shown. The increased autophagy signal further demonstrates the disruption of autophagy in sensitive HMCL.[34]

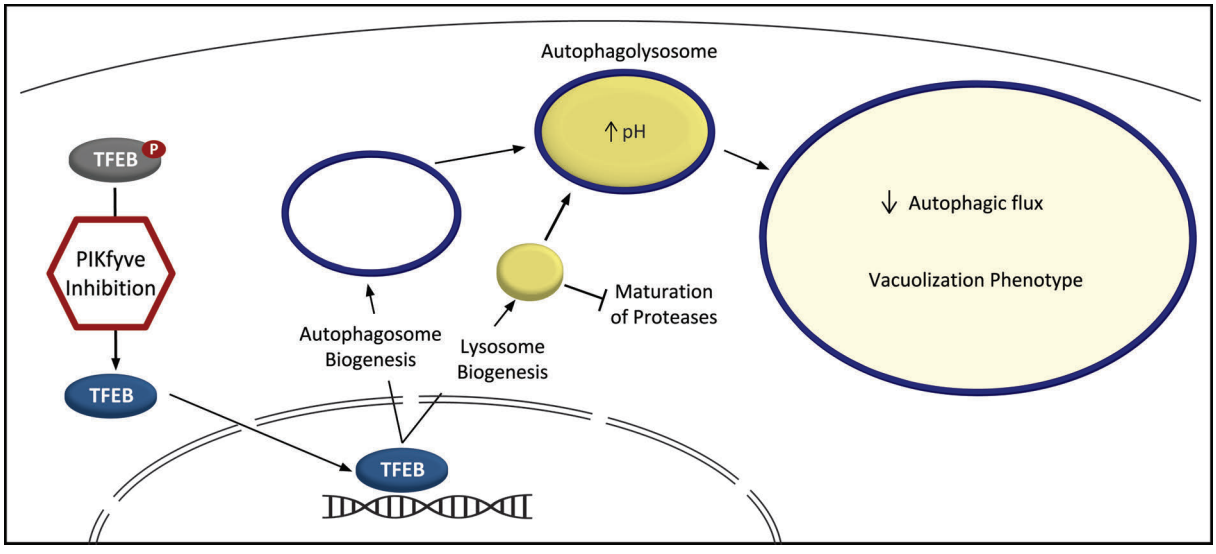

Figure 4. Proposed mechanism of action of PIKfyve inhibitors. PIKfyve inhibition following treatment with APY0201, activates transcription factor EB (TFEB) through dephosphorylation leading to upregulation of autophagosome and lysosome biogenesis. Lysosomal function is disrupted by a decrease in the maturation of proteases and a less acidic pH. Autophagic flux is therefore disrupted, leading to the vacuolization phenotype. We observed that resistant cell lines are able to maintain a more acidic pH and maintain, at least partially, autophagic flux.

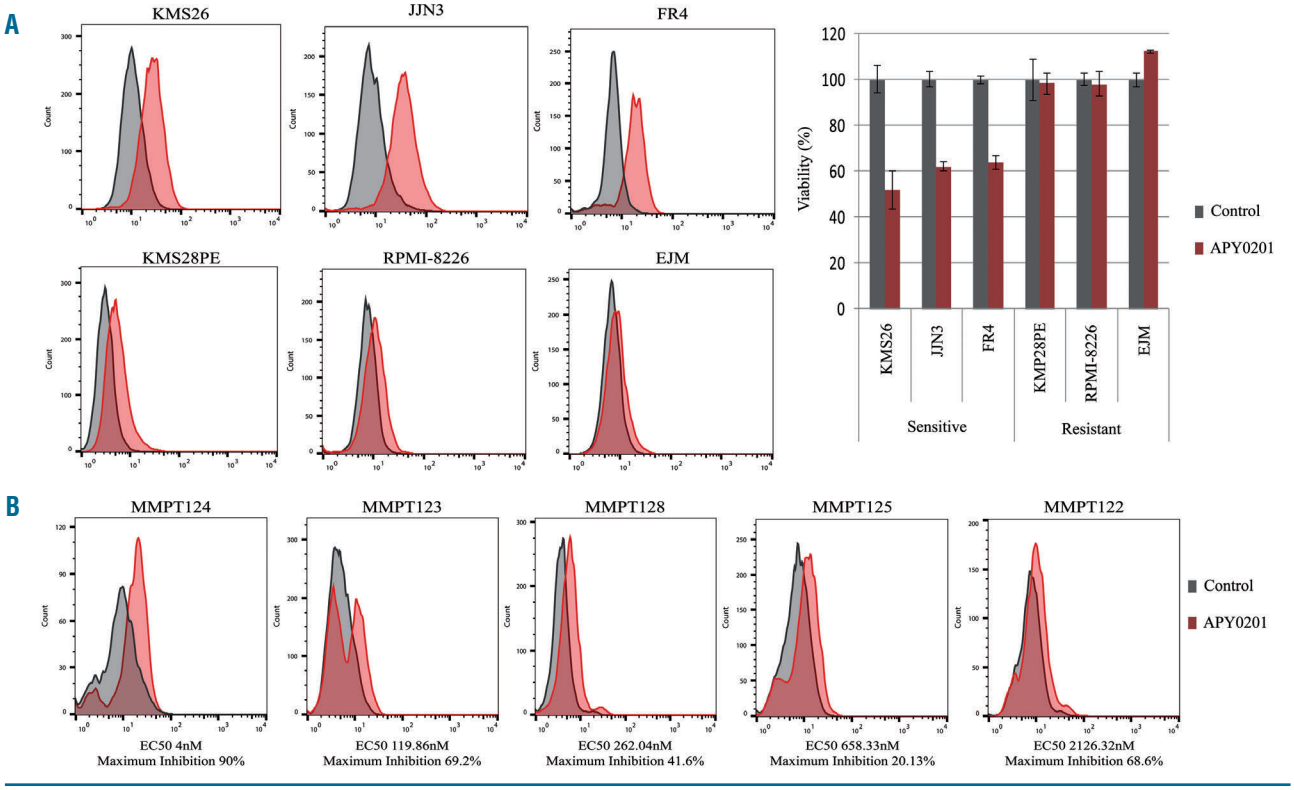

Figure 5. APY0201-mediated increase in autophagic vesicles and cellular toxicity. (A) Three sensitive (KMS26, JJN3, FR4) and three resistant (EJM, KMS28PE, RPMI-8226) human multiple myeloma cell lines treated or not with APY0201 at 100 nM for 18 h. Histogram representation of the green detection reagent detected by flow cytometry and 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT) assay performed following 72 h of incubation with the compound to determine cell viability. (B) The APY0201-mediated increase in autophagic vesicles in primary patients' samples treated or not with the compound for 18 h was inversely proportional to the mid-point half maximal effective concentration (EC_{50}).

Similarly, in primary patients' samples, an APY0201-mediated increase in autophagic vacuoles was inversely proportional to the EC_{50} for the PIKfyve inhibitor APY0201 (Figure 5B). Analogous findings were also seen when HMCL and primary patients' samples were treated for 18 h with apilimod (*data not shown*). This could, therefore, be an efficient and fast assay to predict PIKfyve inhibitor response in MM patients.

Discussion

Although the clinical outcome of MM patients with current therapy protocols has improved markedly,[35,36] most patients ultimately relapse.[37] The identification of novel anti-MM agents therefore remains an important step towards disease control. We first identified the PIKfyve inhibitor APY0201 as a promising anti-MM therapeutic in a preliminary screen and included the PIKfyve inhibitor in a 76-drug panel used for more intensive drug screening for sensitivity. Following 24 h incubation of 25 HMCL and 100 *ex vivo* primary patients' samples with APY0201, this drug was demonstrated to have activity in >90% and 40%, respectively. PIKfyve inhibition was then validated with three different PIKfyve inhibitors, APY0201, YM201636, and apilimod, generating dose-dependent responses in 20 HMCL, while APY0201 and apilimod showed activity in >90% of *ex vivo* patients' samples with a longer 72 h incubation.

The PIKfyve selectivity of apilimod had been demonstrated when this drug was profiled against several kinases and no off-target activity was detected,[12,38] while the specificity of APY0201 was demonstrated against all tested kinases, G-protein-coupled receptors, ion channels, and enzymes, with APY0201 showing superior selectivity over apilimod.[14] YM201636 is less selective, inhibiting PIKfyve and insulin-induced activation of class IA PI3 kinase.[39] Our findings clearly demonstrated increased inhibition of cellular viability with APY0201, when compared to YM201636 and apilimod, in HMCL, NHL cell lines, and primary *ex vivo* patients' samples.

PIKfyve negatively regulates TFEB,[26] and PIKfyve inhibitor sensitivity has been correlated with higher baseline levels of TFEB, a master regulator of the function of lysosomes and autophagy.[29] Following APY0201 treatment, TFEB was found in a dephosphorylated state which correlated with its translocation to the nucleus in representative sensitive and resistant HMCL, as previously noted by others.[12,40] A PtdIns-3,5-P2-dependent regulation of the activation and nuclear translocation of TFEB has also been previously demonstrated.[40] In addition, the endolysosomal swelling phenotype associated with loss of PIKfyve function has been linked to a concomitantly reduced number and increased volume of autophagy organelles after coalescence.[26] These findings clearly link PIKfyve inhibitors with lysosomal and autophagic disruption and were confirmed in our analysis.

Impaired lysosomal degradation of autophagic cargo induced by apilimod driving cell death was suggested in B-NHL.[38] However, we found an accumulation of lysosomal protease precursors, autophagosome and lysosome protein markers, intracellular vacuolization, as well as transcriptomic increases in the lysosome pathway in representative HMCL both sensitive and resistant to APY0201. This indicated that PIKfyve-induced cell death in MM could not be fully explained by the model of apilimod's mechanism of action in B-NHL,[38] whereby PIKfyve inhibition led to impaired lysosomal homeostasis with nuclear translocation of TFEB and vacuole formation, causing cell death. We theorize and provide supporting evidence that one mechanism of resistance to the PIKfyve inhibitor APY0201 in HMCL is by partially maintaining autophagic flux, which decelerates significant imbalances in membrane trafficking and the resulting metabolic alterations.

Autophagy is critical in plasma cell ontogenesis for sustainable immunoglobulin synthesis and endoplasmic reticulum capacity, increasing cellular viability, which may be even more important in plasma cell dyscrasias.[41] Higher basal protein levels of TFEB were shown in HMCL sensitive to APY0201; therefore, since TFEB overexpression has been associated with increased autophagic flux,[42] basal autophagic flux in HMCL could be directly related to the sensitivity to PIKfyve inhibition. MM cells are notably dependent on autophagy for their survival,[43,44] thus targeting autophagy via PIKfyve inhibition could represent an effective treatment option for MM.

A phase 1 clinical trial with apilimod in B-cell malignancies is in progress, with preliminary results showing promising early antitumor activity in heavily pretreated patients and a favorable safety profile at doses of ≤125 mg BID.[45] *Ex vivo* drug screening is suggested to enrich MM patients sensitive to PIKfyve inhibitors in future clinical trials. Detection of autophagy in response to drug exposure is also a promising test to predict sensitivity, although additional validation is necessary. In addition, we anticipate greater sensitivity to PIKfyve inhibitors in patients with trisomies of one or more odd-numbered chromosomes and less sensitivity in MM samples harboring t(11;14).

In summary, we demonstrated promising anti-myeloma activity *in vitro* and *ex vivo* as a result of inhibition of PIKfyve, a novel therapeutic target in MM. PIKfyve inhibitors disrupt lysosomal function and, consequently, autophagic flux, and the high basal necessity of autophagy in plasma cells and MM cells indicate the clinical potential of this novel target in anti-MM strategies. PIKfyve inhibition should be further evaluated in MM.

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