revealed no difference between the three age groups with respect to the total population and the groups with or without rituximab. The hazard ratios (HR) between all age groups were 0.9–1.0 for EFS and 1.1–1.3 for OS, which were not significantly different in all patients and patients treated with or without rituximab. In a multivariate analysis of the treatment given (etoposide and/or rituximab), adjusted for aaIPI risk factors, bulky disease, extranodal involvement >1 and three age groups, which was performed separately for 154 patients from the NHL-B1 trial (CHOEP-14/21 vs CHOP-14/21) and 206 patients from the MINT trial (CHO(E)P-21 ± R), we confirmed that the administration of etoposide (HR = 0.4 with 95% confidence interval (CI) (0.2; 0.7), $P = 0.003$) in the NHL-B1 trial and rituximab (HR = 0.4 with 95% CI (0.2; 0.7), $P = 0.003$) in the MINT trial improved EFS in this AYA population. The administration of rituximab improved also, but not significantly, the OS (HR = 0.4 with 95% CI (0.1; 1.1), $P = 0.075$) in the MINT trial. In conclusion, age up to 35 years was not a risk factor in trials that included young adults aged 18–35 years with aggressive lymphoma treated with CHOP-like regimens with and without rituximab. The EFS and OS results were excellent and were in a range comparable to those achieved using pediatric BFM-type protocols.[1] Therefore, young adults have an excellent outcome when treated with protocols developed for adults. Our data do not support the use of more aggressive protocols used by pediatric oncologists in this age group outside clinical trials. Currently, trials proposed by a European network compare both approaches only in selected ALCL subtypes in patients up to 30 years. We conclude that a prospective comparison of established but rather complicated and more toxic pediatric protocols with the R-CHO(E)P regimen is highly warranted in the AYA age group.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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KH, LT and SZ collected clinical data, analyzed the data and wrote the manuscript. SZ, MZ and ML performed data analyses and gave final approval of the manuscript. GH, GW, NS and MP collected clinical data and gave final approval of the manuscript.

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High-throughput drug screening identifies compounds and molecular strategies for targeting proteasome inhibitor-resistant multiple myeloma

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Proteasome inhibitors (PI) are a cornerstone in the treatment of multiple myeloma (MM).[1] Despite high initial response rates, nearly all MM patients relapse, marking the incurable nature of the disease and the emerging clinical challenge of combating PI resistance. This has created a need for drug discovery approaches that identify new drug cocktails of PIs, such as bortezomib/VELCADE (Btz; Millennium Pharmaceuticals,

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Inc., Cambridge, MA, USA), and other classes of FDA-approved or investigational new drugs. With these objectives in mind, we set out in this study to develop a high-throughput drug screening (HTS) platform to identify chemical structures that selectively kill or re-sensitize PI-resistant MM cells to PIs.

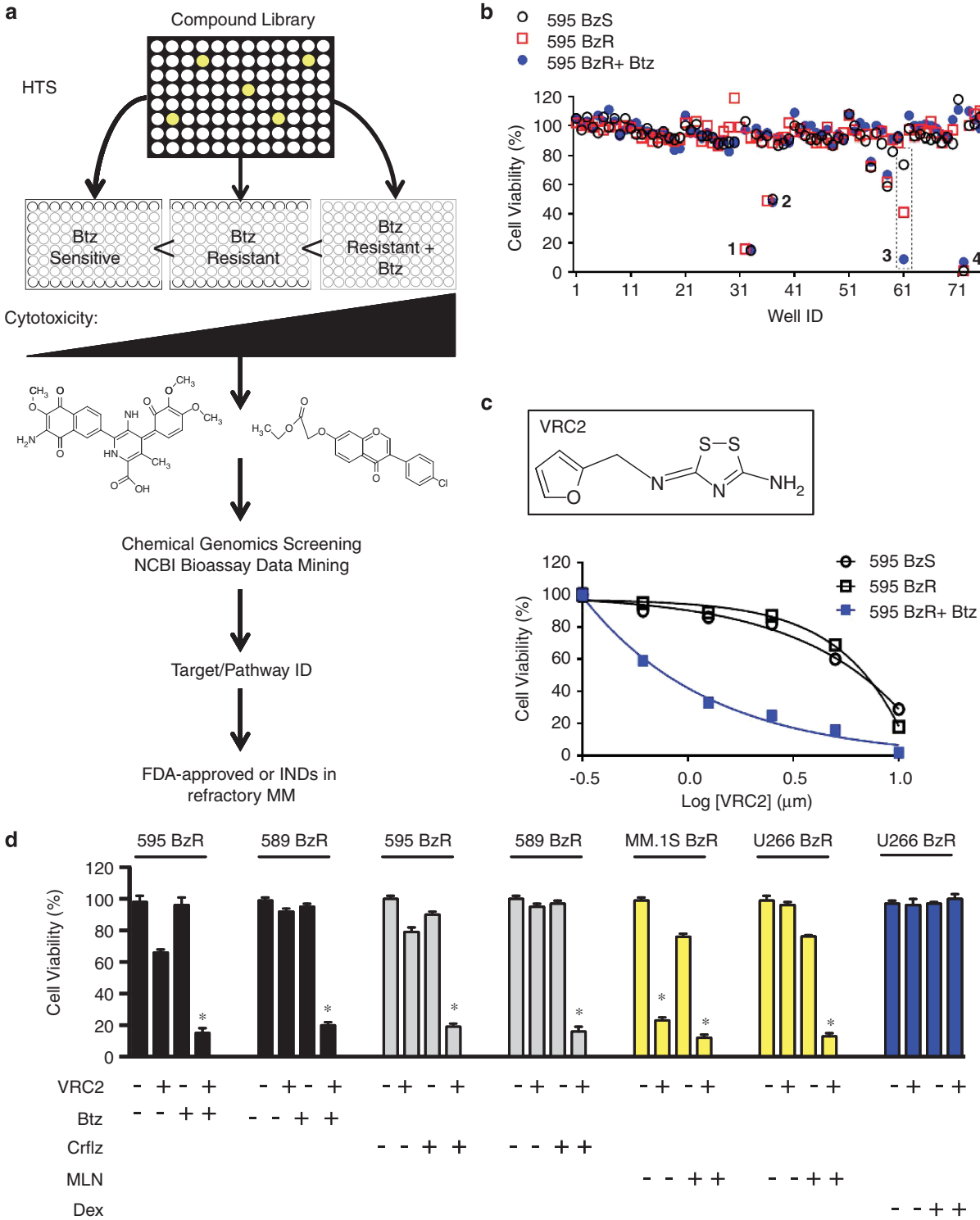
We established a cell-based drug screening assay that incorporated isogenic pairs of Btz-sensitive (BzS) and Btz-resistant (BzR) mouse and human MM cells, which have been described previously[2] and exhibit an approximate 5–7-fold difference in sensitivity to Btz (Supplementary Figure S1).[3] Each plate of library compounds was tested against a plate containing BzS cells, one containing BzR cells, and a third containing BzR cells in the presence of Btz. Cell viability was used as the assay read-out to measure the effects of screened compounds on cell survival and proliferation (Figure 1a and Supplementary Figure S2). It is important to note that the inclusion of the three cell groups allowed us to calculate the relative effects of drugs, and thus identify the structures with selective activity against the resistant cells as single agents or those with the ability to restore Btz sensitivity, rather than those with general cytotoxicity *in vitro* (Figures 1a and b). To demonstrate the utility of this HTS assay in practice, we conducted a pilot round of screening using the NCI Diversity Set II (NCI Developmental Therapeutics Program) of ~1600 small molecules chosen for their core structural diversity and favorably predicted drug-like qualities. In total, our primary screening identified 12 compounds with activity against any of the treatment groups (Supplementary Table S1). Of greater significance, four of the hits showed reproducibly greater activity against BzR cells as single agents or restored sensitivity to Btz in BzR cells when co-treated with Btz. We elected to further pursue compound NSC622608, which we named Velcade Re-sensitizing Compound 2 (VRC2), due to its unknown and potentially novel molecular target/mechanism of action and its ability to synergize with Btz and reverse the resistance phenotype *in vitro* (Figure 1c). We investigated the activity of VRC2 in combination with the other PIs that have been approved for MM (carfilzomib) or are in clinical development (MLN2238). Comparable to what we observed with Btz, VRC2 restored sensitivity to these next- generation PIs in a panel of mouse and human BzR cell lines (Figure 1d). This synergy appeared to be specific for PIs, as VRC2 failed to enhance the sensitivity of MM cells to other classical MM agents including, the glucocorticoid dexamethasone (Figure 1d).

To identify the potential molecular targets of VRC2, we used a combination of data mining and chemical genomics approaches. First, we screened the NCBI PubChem Bioassay Database for published drug screening results that reported VRC2 as a positive hit. We found one report of VRC2 activity against wild-type and mutant forms of the murine double minute 2 (MDM2) E3 ligase, a known regulator of TP53 (p53) activity (PubChem BioAssay identifiers AID: 1442 and 1444 from the Penn Center for Molecular Discovery; Ref. 'A' and 'B').[4,5] Our genomic studies, in which we used kinetic gene expression profiling (GEP) to characterize the gene networks that were induced or repressed following VRC2 treatment, further implicated the p53 pathway as a mechanism of VRC2 action. In human MM.1S BzR cells, kinetic VRC2 pathway analysis showed a strong p53 activation signature ($z = 2.078$; $P = 2.39 \times 10^{-8}$ by Fisher's Exact Test) (Ingenuity Pathway Analysis), which was driven by the upregulation of MT1H, HMOX1 and ANXA2 and downregulation of POLD2, MCM5, MCM4, MCM3, MCM2, KIAA0101 and CCNA2 following VRC2 treatment (Figure 2a and Supplementary Figure S3). Notably, this p53 activation signature was absent in U266 BzR cells, which express an inactivating p53 mutation at codon 161 (Figure 2a).[6] Consistent with the predicted effects of an MDM2 inhibitor, VRC2 increased the expression of p53 in a dose- and time-dependent manner in wild-type p53-expressing MM cell lines (Figure 2b and Supplementary Figure S4A), and induced the expression of p53 target genes *P21*, *PUMA* and *NOXA* (Figure 2c and Supplementary Figure S4B). These mechanistic findings suggested that invoking the p53 pathway by means of MDM2 inhibition is a promising molecular strategy to overcome PI resistance in MM cells. Indeed, when we combined Btz with Nutlin3a, a quintessential MDM2 inhibitor in clinical development,[7] we detected statistically significant synergy in two resistant clones derived from wild-type p53 expressing MM.1S BzR cells (Figure 2d and Supplementary Figure S5A). Interestingly, we

Figure 1. HTS platform for discovering PI-sensitizing compounds. (**a**) The schema shown outlines the flow of drug library screening and follow-up data mining and chemical genomics approaches that were used to identify the molecular mechanism of action of confirmed hits. The cell-based assay was adapted for high throughput multi-well format and made use of isogenic pairs of PI-sensitive and -resistant mouse or human cell lines that were established by our group and described previously. Library compounds were screened against three groups of cells: (1) PI sensitive; (2) PI resistant; and (3) PI resistant in the presence of 20 nM Btz, a concentration that is highly toxic to sensitive, parental cells but ineffective against the PI-resistant population. The bioluminescence-based Cell TiterGlo (Promega Corporation, Fitchburg, WI, USA) cell viability assay was chosen as the HTS read-out due to the high sensitivity and quantitative nature of this system. A Z' factor of 0.49 was calculated using Btz (20 nM) as a positive control and DMSO as a negative control in 595 BzS mouse MM cells. Compounds that exhibited preferential killing of BzR cells compared to BzS cells and those that synergized with Btz in the BzR cells were selected for secondary screening in panels of mouse and human BzR cells. Confirmed hits were then analyzed by chemical genomics to determine potential target pathways and mechanisms of action. Additional mechanistic leads were acquired by searching the publicly available NCBI Bioassay Database for reports of activity against known molecular targets in archived drug screening data sets. Identification of the drug molecular mechanism could be used in the development of investigational new drugs, or matched to existing FDA-approved agents that could be expeditiously tested in trials of refractory MM. In our study, the unknown compound, VRC2 (NSC622608), was presented and discussed as an example of a molecular probe/tool compound that was discovered and characterized using this platform and then used to implicate a developmentally advanced drug candidate (i.e., Nutlin3a). (**b**) Raw HTS data from a representative plate (NCI Diversity Set II plate number 4662) are shown. The data points from individual compounds that would have been selected as positive hits are denoted by numbers 1–4. Compounds 1, 2 and 4 show general cytotoxic activity as these chemical structures target BzS and resistant BzR cells without selectivity. By comparison, hit number 3 would have been given priority in follow-up studies due to its greater potency for killing the BzR cells and ability to re-sensitize BzR cells to Btz. The precise chemical identity of hit number 3 is camptothecin, a known topoisomerase inhibitor. The full list of positive hits from the primary screening is found in Supplementary Table S1. (**c**) The chemical structure of the experimental compound VRC2 is shown. In secondary layers of screening, a dose range of VRC2 was used to treat 595 BzS, 595 BzR and 595 BzR cells in the presence of Btz (25 nM). Normalized cell viability data are shown. VRC2 reduced cell viability with equal potency in the BzS and BzR cells as a single agent; however, it showed robust synergy when combined with Btz in BzR cells (blue dose-response curve). Note that because Btz alone had no effect on the viability of BzR cells, the separation of the VRC2-alone curve and the combination curve is indicative of a superadditive/synergistic drug interaction. (**d**) Additional follow-up experiments were conducted using panels of mouse (595 and 589) and human (MM.1S and U266) BzR cell lines along with the next-generation PIs carfilzomib (Crflz) and MLN2238 (MLN). The indicated cell lines were treated with VRC2 (2.5 μM) and Btz (25 nM) or carfilzomib (25 nM) or MLN2238 (55 nM), alone or in combination. Cell viability data are shown (*$P < 0.01$, $N = 3$). We detected no synergy between VRC2 and the glucocorticoid dexamethasone (Dex; 10 μM).

found that the combination of carfilzomib and Nutlin3a was highly synergistic and significantly more robust than the combination with Btz (Figure 2d and Supplementary Figure S5A). While the addition of Nutlin3a to Btz treatment increased the percentage of apoptotic cells by 8–10% ($P=0.05$, $N=4$), the combination of Nutlin3a and carfilzomib increased apoptosis by 35–45% ($P=0.004$, N = 4; Figure 2e). Similar results were observed using resistant mouse cell models 595 BzR (Supplementary Figure S5B) and 589 BzR (data not shown), both of which express wild-type p53.[2] In mutant p53-expressing U266 BzR cells, synergy between Nutlin3a and PIs was also evident, albeit requiring higher concentrations of Nutlin3a (Figure 2f), and, consistent with the wild-type p53 models, the synergy was more pronounced with carfilzomib than Btz.

In this study, we have described and implemented a cell-based HTS platform for discovering the drugs and molecular mechanisms that specifically target treatment-resistant MM cells. The cell-based approach is advantageous for two reasons. First, given the multiple known and unknown mechanisms by which cells acquire



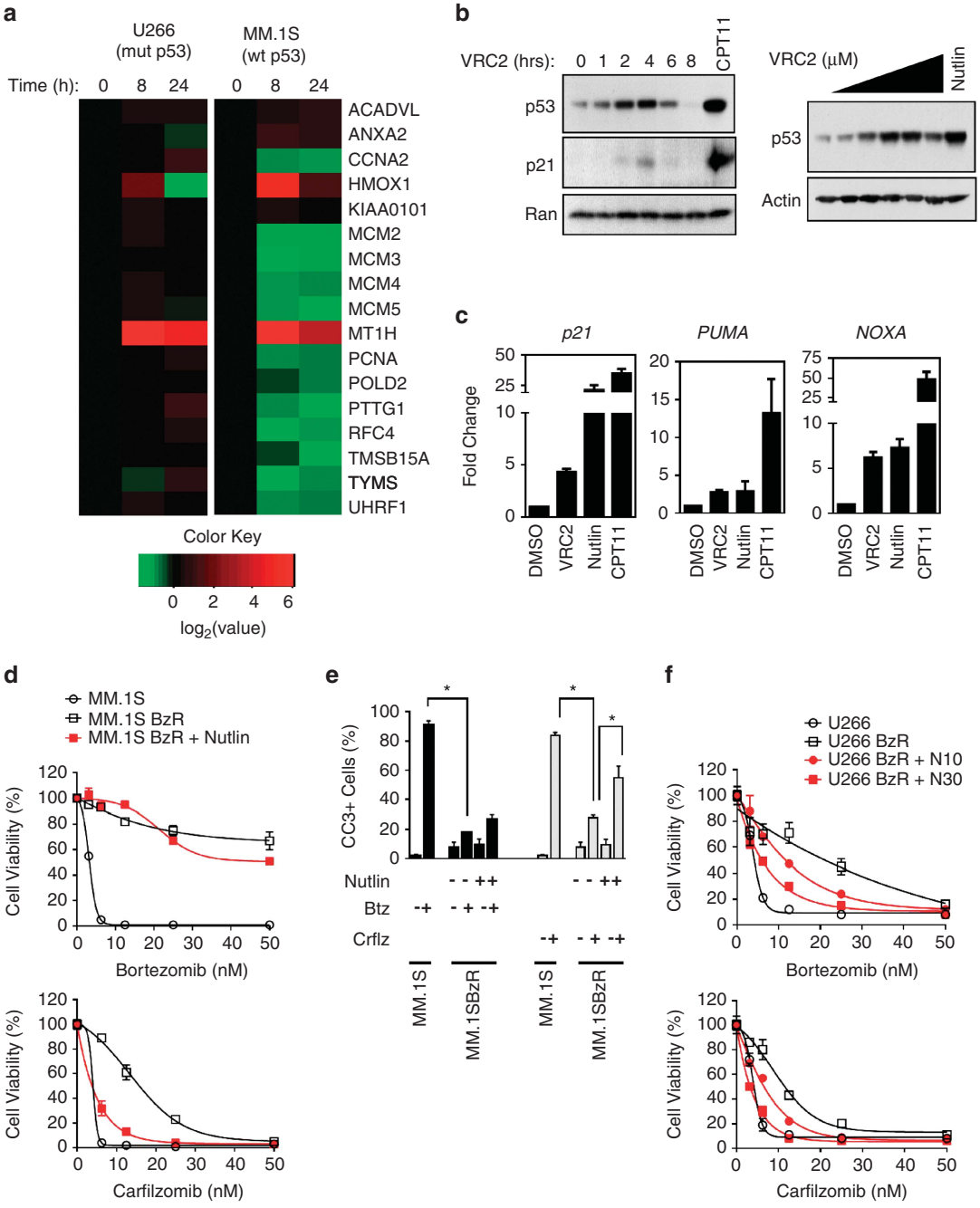


Figure 2. Chemical genomic screening identifies MDM2 inhibition as a promising molecular strategy for overcoming PI resistance in MM cells. (**a**) Human MM.1S BzR cells were treated with a fixed dose of 300 nM VRC2 and collected for gene expression analysis at 0, 8 and 24 h following treatment. The kinetic gene expression patterns of all genes showed evidence of p53 pathway activation by the downstream profiles of the 17 genes shown in the heatmap (Ingenuity Pathway Analysis). Similar experiments were conducted using mutant p53-expressing U266 BzR cells, and as expected, the p53 pathway activation signature was absent in these p53-deficient cells. (**b**) VRC2 induces molecular effects that are characteristic of MDM2 inhibition. Wild-type p53-expressing MM.1S BzR cells were treated with 500 nM VRC2 for the indicated time points (top) or treated with increasing concentrations of VRC2 (0, 0.125, 0.25, 0.5, 1.0, 2.0 μM) for a fixed time point (4 h). Western blots are shown. CPT-11 (33 μM) and Nutlin3a (5 μM) were included as positive controls. (**c**) MM.1S BzR cells were treated with VRC2 (500 nM), Nutlin3a (5 μM) or CPT-11 (33 μM) for 4 h. qPCR analysis was conducted for the indicated p53 target genes. (**d**) Resistant MM.1S BzR cells were treated with a dose range of Btz (top) or Crflz (bottom) for 24 h in the presence or absence of Nutlin3a (5 μM). Cell viability data are shown. There was no effect of single agent Nutlin3a at this time point and therefore any separation of the curves indicates a synergistic drug interaction. Parental MM.1S cells, which are highly sensitive to Btz and Crflz, are shown for comparison. (**e**) MM.1S BzR cells were treated with Btz (25 nM) or Crflz (25 nM) as single agents and in combination with Nutlin3a (5 μM). Cells were treated for 24 h, then fixed and stained for cleaved/active caspase-3 (CC3), and the percentage of CC3-positive (CC3+) cells were quantified by flow cytometry. Parental/sensitive MM.1S cells are shown for comparison (*$P < 0.01$ by *t*-test, $N = 4$). (**f**) Resistant U266 BzR cells were treated with a dose range of Btz (*top*) or Crflz (bottom) for 24 h in the presence or absence of 10 μM Nutlin3a (N10) or 30 μM Nutlin3a (N30). Cell viability data are shown. There was no effect of single-agent Nutlin3a at this time point and therefore any separation of the curves indicates a synergistic drug interaction. Parental U266 cells, which are highly sensitive to Btz and Crflz, are shown for comparison.

resistance to PIs,[8–10] a cell-based approach removes molecular bias. Second, the use of isogenic BzS and BzR cell models provides a relative assessment of cytotoxicity and the opportunity to identify compounds that selectively target PI-resistant populations. As proof of concept, we conducted a small-scale drug screen from which we identified VRC2, a compound with the ability to restore PI sensitivity to resistant MM cells. While VRC2 is not an ideal candidate for further development due to characteristics that predict unfavorable physiochemical properties (that is, furan and diathiazol toxicophores, a high number of hydrogen bond acceptors at 13, and predicted high clearance risk), in our study, it effectively served as a molecular probe to demonstrate the utility of coupling HTS with other approaches such as chemical genomics to pinpoint molecular mechanisms and therapies that are already approved for clinical use or are in human trials. Our investigation into the mechanism of VRC2 implicated MDM2 inhibition and p53 pathway activation as molecular strategies for targeting PI-resistant cells, findings that directed us toward the more clinically advanced MDM2 inhibitor, Nutlin3a. MDM2 inhibition has been shown by others to enhance the activity of Btz,[11–13] which supports the ability of our method to identify bona fide druggable mechanisms for overcoming PI resistance. Our study further demonstrates that Nutlin3a not only augments the cytotoxic activity of Btz in Btz naive cells, but can also restore sensitivity to Btz once MM cells have acquired therapeutic resistance. It is also noteworthy that we found the combination of Nutlin3a/carfilzomib to be substantially more synergistic than Nutlin3a/Btz, findings that require further investigation but carry potentially significant translational implications. The mechanistic basis for this difference is not clear, however, it may be explained by the known differences in binding kinetics between the two PIs—inhibition of the 26S proteasome by Btz is reversible, whereas carfilzomib binds irreversibly. In summary, this proof-of-concept work validates the utility of our HTS approach for discovering lead compounds and uncovering molecular mechanisms for targeting PI-resistant MM, and provides a platform and the impetus for larger-scale screening efforts.

CONFLICT OF INTEREST

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Does ruxolitinib improve survival of persons with MPN-associated myelofibrosis? Should it?

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JAK2-activating mutations are linked to development of myeloproliferative neoplasms (MPNs), a discovery that revolutionized the therapy of persons with MPN-associated myelofibrosis. Targeting the JAK/STAT pathway with ruxolitinib, a JAK1/JAK2 inhibitor, suppresses heamatopoiesis and pro-inflammatory cytokines, reduces splenomegaly and disease-related symptoms.[1–4] These effects are not specific for the neoplastic clone and the response rates are similar in persons with and without the $JAK2^{V617F}$ and other JAK2 mutations.[5] Based on these data, ruxolitinib was approved by the US Food and Drug Administration (FDA) for therapy of splenomegaly in persons with

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