PYK2/FAK inhibitors reverse hypoxia-induced drug resistance in multiple myeloma

Integrins and downstream signaling kinases, such as proline-rich tyrosine kinase 2 (PYK2) and focal adhesion kinase (FAK), are important for sensing cell adhesion to the extracellular matrix and were demonstrated to be involved in multiple myeloma (MM) progression, dissemination and drug resistance. We found that the dual PYK2/FAK inhibitors, VS-4718 and VS-6063 (defactinib), antagonized hypoxia-induced resistance of myeloma to proteasome inhibitors both *in vitro* and *in vivo*.

MM is the malignancy of plasma cells accumulating mainly in the bone marrow and is the second most common hematologic malignancy. The understanding of molecular mechanisms of cell signaling pathways involved in myeloma has led to the development of new targets and therapies, with a consequent significant improvement of MM treatment. However, nearly all MM patients relapse, which is mainly attributed to development of drug resistance and recurrence due to minimal residual disease (MRD).[1,2]

The interaction between MM cells and their tumor microenvironment was shown to play a crucial role in drug resistance in MM.[3-5] One of the major routes of interaction between MM cells and the tumor microenvironment is cell-cell interactions and interactions with the extracellular matrix.[3-5] Integrins are transmembrane proteins that attach to other cells as well as the extracellular matrix to facilitate cell adhesion. There is growing evidence demonstrating that focal adhesion kinase proteins including PYK2 and FAK play a pivotal role in integrin signaling in tumors, especially in progression and drug resistance in MM.[5-9] *PYK2* expression was reported to be increased in plasma cells harvested from patients with monoclonal gammopathy of undetermined significance, smoldering MM, as well as MM compared to the level in plasma cells from healthy individuals, suggesting its role in promoting MM progression.[6] Moreover, PYK2 overex-

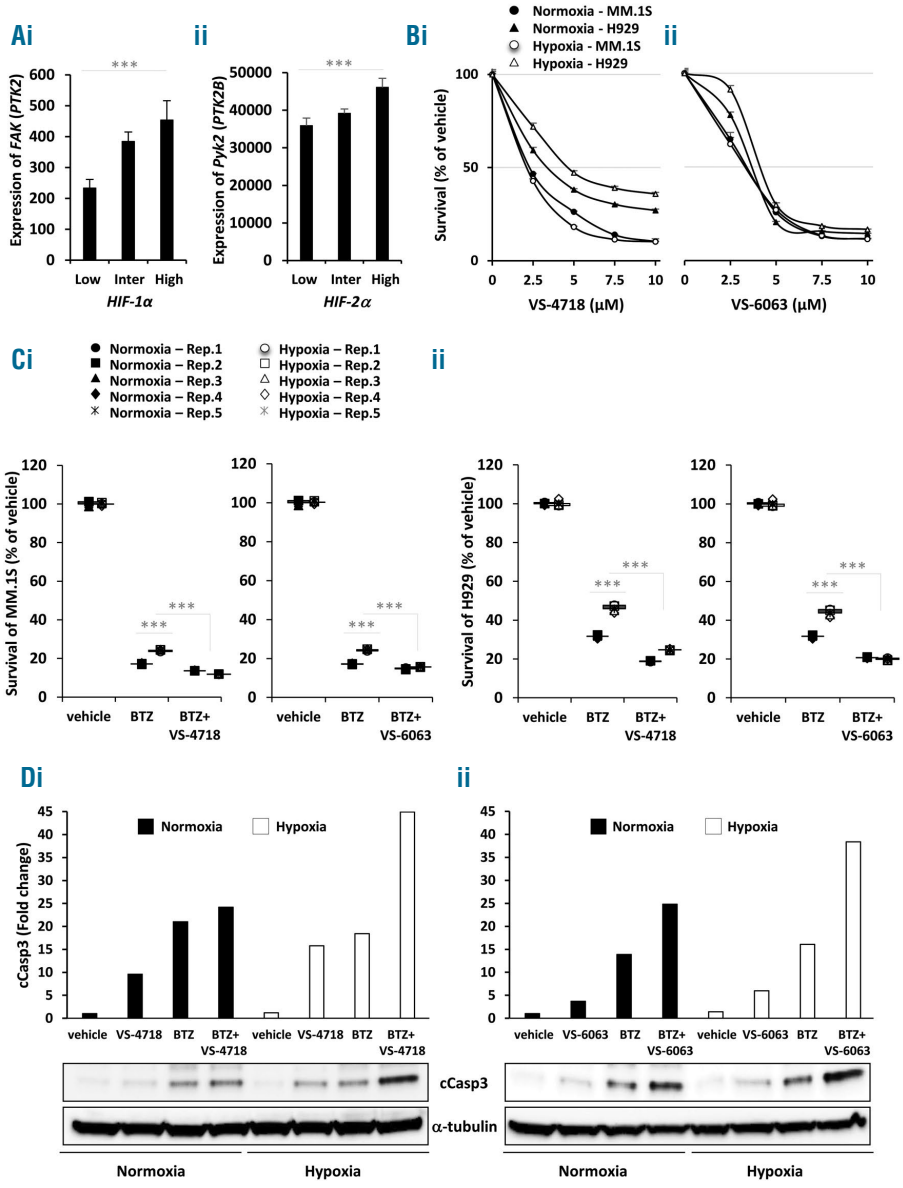

Figure 1. The effect of PYK2/ FAK inhibition on hypoxia-induced drug resistance in multiple myeloma, *in vitro*. Correlation of the gene expression of (Ai) *FAK* and *HIF-1α*, and (Aii) *PYK2* and *HIF-2α* in primary multiple myeloma (MM) plasma cells. Survival of MM.1S and H929 cells treated with increasing concentrations of (Bi) VS-4718 and (Bii) VS-6063 for 24 h in normoxic (21% O_2) and hypoxic (1% O_2) conditions based on a survival/cytotoxic MTT assay. Survival of (Ci) MM.1S cells treated with VS-4718 (2.5 μM) or VS-6063 (2.5 μM) in combination with bortezomib (5 nM) and (Cii) H929 cells treated with VS-4718 (2.5 μM) or VS-6063 (2.5 μM) in combination with bortezomib (2 nM) for 24 h in normoxic and hypoxic conditions based on a survival/cytotoxic MTT assay. Apoptosis of (Di) MM.1S cells treated with VS-4718 (2.5 μM) or (Dii) VS-6063 (2.5 μM) with and without bortezomib (5 nM), demonstrated as cleaved caspase-3 using immunoblotting of the protein with densitometric analysis, and normalized to α-tubulin (a representative image). Results are shown as mean ± standard deviation (SD) of five independent biological replicates and repeated minimum in three separate experiments; the statistical significance was assessed using the Student *t*-test or ANOVA (***P<0.001). cCasp3: cleaved caspase-3. BTZ: bortezomib.

pression in MM cells increased tumor growth and decreased mice survival, whereas PYK2 inhibition led to a reduction of MM tumor growth *in vivo*, again indicating its role in MM progression.[6]

We have previously shown that regions of hypoxia (decreased oxygenation) develop in the bone marrow niche during MM progression,[10,11] and that hypoxia plays a major role in the development of a cancer stem cell-like phenotype and induction of drug resistance observed in MRD in MM.[12-14] We hypothesized that inhibiting PYK2/FAK, using VS-4718 and VS-6063, would antagonize hypoxia-induced drug resistance of MM cells.

To test our hypothesis, first we analyzed the correlation between intrinsic markers of hypoxia and the expression of *FAK* and *PYK2*. Both hypoxia-inducible factor (HIF)-1α and HIF-2α isoforms are negative prognostic factors for treatment efficacy and MM patient survival, and are frequently used as hypoxic markers.[13] We therefore analyzed the expression of *FAK (PTK2)* and *PYK2 (PTK2B)* and correlated their levels with those of *HIF-1α* and *HIF-2α* in plasma cells from patients with MM in the CoMMpass trial,[15] with a dataset from a total of 724 newly diagnosed MM patients included in the analysis. We found that *FAK* expression correlated with *HIF-1α* expression (Figure 1Ai), while *PYK2* expression correlated with that of *HIF-2α* (Figure 1Aii), which suggests that both FAK and PYK2 are regulated in hypoxia.

We therefore investigated the role of PYK2/FAK inhibition on the proliferation and hypoxia-induced drug resistance in MM. First, we treated MM cells with increasing doses of dual PYK2/FAK inhibitors (VS-4718 and VS-6063, provided by Verastem, Inc., Needham, MA, USA) as single agents, and analyzed the survival of MM cells, MM.1S and H929, in normoxia (21% O_2) and hypoxia

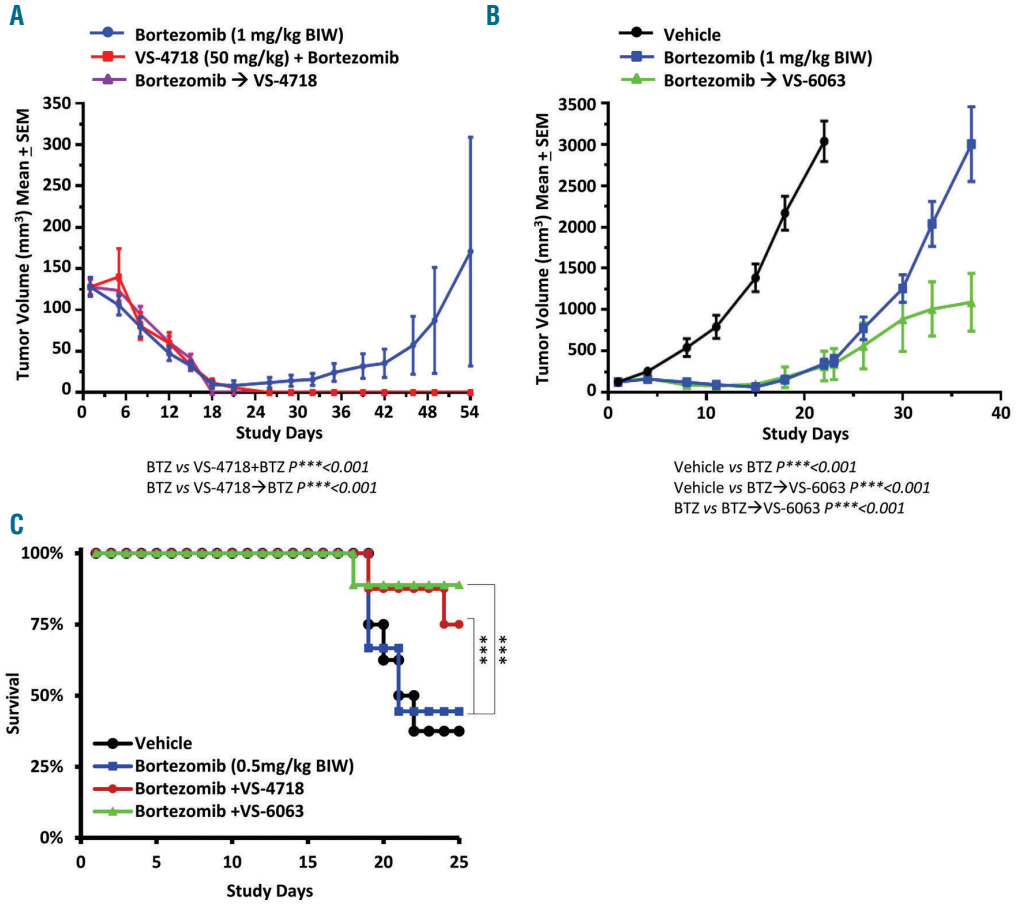

Figure 2. The effect of PYK2/ FAK inhibition on hypoxia-induced drug resistance in multiple myeloma, *in vivo*. (A) The effect of VS-4718 ± bortezomib on tumor volume tested in a subcutaneous mouse model. When H929 tumors reached a mean volume of ~125 mm^3, mice were randomized into three groups (n=10 per group) and treated with: (i) bortezomib (1 mg/kg, biweekly) alone; (ii) the combination of VS-4718 (50 mg/kg, BID) and bortezomib concurrently; and (iii) first bortezomib to simulate minimal residual disease (MRD) followed by VS-4718 (sequentially). Tumor growth was measured twice weekly using calipers and is shown as the mean ± standard error of mean (SEM). (B) The effect of VS-6063 ± bortezomib on relapse (tumor growth) tested in a subcutaneous mouse model. When H929 tumors reached a mean volume of ~125 mm^3, mice were randomized into three groups (n=10 per group) and treated with: (i) vehicle; (ii) bortezomib (1 mg/kg, biweekly); and (iii) first bortezomib to simulate MRD followed by VS-6063 (50 mg/kg, BID) (sequentially). Tumor growth was measured twice weekly using calipers and is shown as the mean ± SEM. (C) The effect of VS-4718 and VS-6063 combined with bortezomib on mice survival tested in a disseminated xenograft mouse model; MM.1S-Luc-GFP cells were injected intravenously into SCID mice and tumors were allowed to grow for 3 weeks, after which tumor growth was determined using bioluminescent imaging. All mice were then treated with bortezomib (1 mg/kg) for 2 weeks to induce MRD. The mice were randomized into four groups (n=9 per group) and treated with: (i) vehicle; (ii) bortezomib alone (0.5 mg/kg, biweekly); (iii) a combination of VS-4718 (50 mg/kg, BID) and bortezomib; and (iv) a combination of VS-6063 (50 mg/kg, BID) and bortezomib. Survival was assessed daily and is demonstrated as a Kaplan–Meier curve. Statistical analysis was performed using the Student *t*-test and values were considered significantly different for *P* values less than 0.05 (*P****<0.001). BTZ: bortezomib; BIW: biweekly.

(1% O_2) using the MTT assay. We found that MM cells had similar responses in both hypoxia and normoxia, with a half maximal inhibitory concentration (IC_{50}) of 2.5-5 μM for the two inhibitors in the two cell lines (Figure 1B). Despite the upregulation of pPYK2 in hypoxia (*Online Supplementary Figure S1A*), no differences in the sensitivity of the MM cells to the drugs was observed in hypoxia (Figure 1B).

We previously have demonstrated that hypoxia induced resistance to proteasome inhibitors, including bortezomib and carfilzomib, in MM cells.[12] Thus, we tested the effect of the PYK2/FAK inhibitors on the hypoxia-induced resistance to bortezomib and carfilzomib in MM cells. We treated MM cells with bortezomib in combination with VS-4718 or VS-6063 in normoxia or hypoxia, and analyzed the effect of this combination on MM cell survival using MTT and apoptosis assays. Hypoxia induced resistance to bortezomib, as we previously demonstrated,[12] and the combination treatment with both PYK2/FAK inhibitors re-sensitized MM cells to bortezomib *in vitro*, significantly decreasing the survival of MM.1S (Figure 1Ci; *Online Supplementary Figure S2Ai*) and H929 (Figure 1Cii; *Online Supplementary Figure S2Aii*) according to the results of the MTT assay (both normalized to untreated and raw values). VS-4718 (Figure 1Di) and VS-6063 (Figure 1Dii) also increased apoptosis and caspase-3 cleavage in MM cells, in response to bortezomib. In addition, we used the Chou-Talalay method to calculate combination indexes and found that bortezomib in combination with either VS-4718 or VS-6063 had an additive effect on MM cell survival (*data not shown*). Similarly, we demonstrated that PYK2/FAK inhibitors (VS-4718 and VS-6063) overcame hypoxia-induced resistance to carfilzomib in MM.1S (*Online Supplementary Figure S3Ai, Bi*) and H929 cell lines (*Online Supplementary Figure 3Aii, Bii*), both normalized to untreated and raw values. Combination index calculations showed that carfilzomib with VS-4718 had an additive effect, while carfilzomib with VS-6063 had a synergistic effect on MM cell survival (*data not shown*).

Next, we tested the effect of PYK2/FAK inhibitors on tumor progression in localized and disseminated mouse models simulating MRD, *in vivo*. Approval for all *in vivo* studies was obtained from the Ethical Committee for Animal Experiments at Washington University in St. Louis Medical School. In the first *in vivo* model, H929 cells were injected subcutaneously and the treatment started when tumor volume reached an average of 125 mm^3. All animals were treated with bortezomib for only 18 days during which tumor size reduced to a minimum detectable, recapitulating the complete remission occurring in MM patients and simulating MRD. At day 18 (when the size of the tumor was unmeasurable), animals were randomized to three groups: (i) a group which continued to receive bortezomib only; (ii) a group which received bortezomib concurrently with VS-4718; and (iii) a group which received VS-4718 alone. Mice treated only with bortezomib developed drug resistance and relapsed over the following 6 weeks with tumor size returning to similar to that before treatment. In the other two groups, VS-4718 alone or a combination of VS-4718 and bortezomib prevented development of MM in the mice (Figure 2A). In addition, H929 cells were injected subcutaneously and the treatment started when the tumor volume reached an average of 125 mm^3. The mice were then randomized to three groups and treated with: (i) vehicle; (ii) bortezomib alone; or (iii) sequential therapy with bortezomib for 16 days followed by VS-6063 alone after day 16 (when the size of the tumor was unmeasurable). Compared to treatment with the vehicle, treatment with bortezomib significantly delayed tumor progression, but the tumor size reached similar volume. In the third group, sequentially administered VS-6063 after bortezomib treatment cessation, tumor progression was significantly delayed, and at day 38, the average tumor volume was three times smaller than that in the bortezomib-treated group (Figure 2B). These results indicate that VS-4718 prevented, while VS-6063 delayed tumor relapse in a subcutaneous MM model.

To highlight the role of the tumor microenvironment in the bone marrow, we utilized a disseminated xenograft mouse model in which MM.1S cells were injected intravenously into SCID mice. Tumor progression was allowed over the course of 3 weeks, then animals were treated with bortezomib alone for 2 weeks in order to develop MRD and resistance to bortezomib. The mice were then randomized into four groups that received: (i) vehicle; (ii) bortezomib alone; (iii) a combination of bortezomib with VS-4718; or (iv) a combination of bortezomib with VS-6063. The survival of the mice was monitored daily. Survival of mice treated with vehicle was comparable to that of the mice treated with bortezomib; however, both PYK2/FAK inhibitors combined with bortezomib significantly prolonged survival of the animals in the disseminated MM mouse model (Figure 2C).

In conclusion, we found that single-agent treatment with the PYK2/FAK inhibitors, VS-4718 and VS-6063 (defactinib), decreased proliferation and increased apoptosis of MM cells. More importantly, the inhibitors re-sensitized MM cells to hypoxia-induced resistance to proteasome inhibitors, bortezomib and carfilzomib, both *in vitro* and *in vivo*. Moreover, the PYK2/FAK inhibitor VS-4718 was able to prevent relapse in an *in vivo* MM model simulating MRD. Overall, our findings demonstrate that targeting PYK2/FAK using small molecule inhibitors directly affected MM cancer cells, also in the presence of tumor microenvironment (including the hypoxic component). Moreover, PYK2/FAK inhibitory activity was enhanced in combination with proteasome inhibitors, suggesting the crucial role of inhibiting PYK2/FAK in rendering tumor responsiveness to therapies. These data provide a basis for future clinical trials on sensitizing relapsed/refractory MM patients to therapy with PYK2/FAK inhibitors and on using these drugs to reduce relapse after frontline treatment in an MRD setting. VS-6063 (defactinib) is being evaluated in ongoing clinical trials for patients with multiple types of cancer.

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