Small Molecule Therapeutics

BET Protein Antagonist JQ1 Is Synergistically Lethal with FLT3 Tyrosine Kinase Inhibitor (TKI) and Overcomes Resistance to FLT3-TKI in AML Cells Expressing FLT-ITD

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Abstract

Recently, treatment with bromodomain and extraterminal protein antagonist (BA) such as JQ1 has been shown to inhibit growth and induce apoptosis of human acute myelogenous leukemia (AML) cells, including those expressing FLT3-ITD. Here, we demonstrate that cotreatment with JQ1 and the FLT3 tyrosine kinase inhibitor (TKI) ponatinib or AC220 synergistically induce apoptosis of cultured and primary CD34+ human AML blast progenitor cells (BPC) expressing FLT3-ITD. Concomitantly, as compared with each agent alone, cotreatment with JQ1 and the FLT3-TKI caused greater attenuation of c-MYC, BCL2, and CDK4/6. Simultaneously, cotreatment with JQ1 and the FLT3-TKI increased the levels of p21, BIM, and cleaved PARP, as well as mediated marked attenuation of p-STAT5, p-AKT, and p-ERK1/2 levels in AML BPCs. Conversely, cotreatment with JQ1 and FLT3-TKI was significantly less active against CD34+ normal bone marrow progenitor cells. Knockdown of BRD4 by short hairpin RNA also sensitized AML cells to FLT3-TKI. JQ1 treatment induced apoptosis of mouse Ba/F3 cells ectopically expressing FLT3-ITD with or without FLT3-TKI-resistant mutations F691L and D835V. Compared with the parental human AML FLT3-ITD–expressing MOLM13, MOLM13-TKIR cells resistant to AC220 were markedly more sensitive to JQ1-induced apoptosis. Furthermore, cotreatment with JQ1 and the pan-histone deacetylase inhibitor (HDI) panobinostat synergistically induced apoptosis of FLT3-TKI-resistant MOLM13-TKIR and MV4-11-TKIR cells. Collectively, these findings support the rationale for determining the in vivo activity of combined therapy with BA and FLT3-TKI against human AML cells expressing FLT3-ITD or with BA and HDI against AML cells resistant to FLT3-TKI.

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Introduction

FMS-like tyrosine kinase 3 (FLT3) is a member of the class III receptor tyrosine kinase (TK) family that is expressed on the cell membrane of most acute myelogenous leukemia (AML) blast progenitor cells (BPC; refs. 1 and 2). Interaction with FLT3 ligand causes homodimerization, autophosphorylation, and activation of FLT3 (1, 2). Activated FLT3 transduces progrowth and prosurvival signaling through STAT5, PI3K/AKT, and RAS/RAF/ERK1/2 pathways (2, 3). Activating, somatic, gain-of-function FLT3 internal tandem duplication (ITD) mutations and FLT3-TK domain (TKD) mutations are observed in approximately 25% and 8% of AML, respectively (2, 3). In addition to FLT3-TKD-D835V/Y, other mutations within the TKD have also been reported (4). Several FLT3 kinase inhibitors (FLT3-TKI) have been clinically tested but none is yet approved for the therapy of AML (5, 6). Although treatment with FLT3-TKI has been documented to induce clinical remissions, emergence of resistance that prevents durable remissions and limits leukemia-free survival remains a challenge (6). Although approved for BCR-ABL TKI-resistant CML, ponatinib has also been shown to potently inhibit FLT3 and its downstream signaling and exert preclinical and clinical activity against AML-expressing FLT3-ITD (6, 7). AC220 (quizartinib) is a highly active FLT3-TKI, which induces terminal myeloid differentiation in vivo and is currently being evaluated for efficacy in clinical trials (6, 8–10). Recently, ponatinib was shown to be active against AC220-resistant kinase domain mutants of AML-expressing FLT3-ITD (6, 11). Several studies have documented the mechanisms of resistance to FLT-TKI in AML blasts expressing FLT3 mutation (2, 4, 6, 12–15). These include the acquisition of secondary FLT3-TKD mutations: the gatekeeper F691L in the TKD1 of the ATP binding pocket, the D835V/Y/F in the activation loop of TKD2, and the compound FLT3-ITD/
The F69I mutation (4, 6, 14). FLT3-TKI resistance may also be because of amplification of the FLT3 locus on chromosome 13 with overexpression of FLT3-ITD protein (2). Recently, increased FLT3 ligand (FL) expression with upregulation of STAT3 activity and survivin levels has been noted in AML blasts, demonstrating resistance to FLT3-TKI that otherwise did not exhibit mutations in FLT3-TKD (15–17). Levels of FL have been demonstrated to rise during treatment of AML with chemotherapy, and FLT3 ligand was shown to impede the in vitro and in vivo efficacy of FLT3-TKI (15–17). Thus, the autocrine induction of FL may mediate activation of FLT3-ITD and confer clinical resistance to FLT3-TKIs (15–17). Finally, increase in the activity of the compensatory and collateral progrowth and prosurvival signaling pathways and upregulation of antiapoptotic proteins may also confer resistance to FLT3-TKI treatment (2, 6, 13). Collectively, these observations underscore the need to develop and test novel agents and combinations that are more effective, as well as overcome resistance to FLT3-TKI in AML BPCs.

The family of bromodomain and extraterminal (BET) proteins, including BRD4, are chromatin "reader" proteins that contain the N-terminal double, tandem, 110 amino acids long bromodomains, through which they bind to the acetylated lysines on histone proteins (18, 19). Through their extraterminal (ET) protein-interacting domain in the C-terminus, BET proteins recruit coregulatory complexes containing chromatin-modifying enzymes, chromatin remodeling factors, and the mediator elements to the chromatin for regulating gene transcription (19–21). The C-terminal domain (CTD) of BRD4 interacts with positive transcription elongation factor b (pTEFb), the heterodimer composed of cyclin T and CDK9, which phosphorylates serine 2 on the CTD of RNA pol II (RNAP2) causing its pause release and mRNA transcript elongation (20–23). Recently, an RNAi screen identified BRD4 as an effective and promising target in human AML (24). Several structure/activity-based BET protein small molecule inhibitors have been developed, for example, JQ1 and I-BET151 (25, 26). Treatment with JQ1 disrupts the binding of the bromodomains of BRD4 to acetylated lysines, displacing the BET proteins, along with the associated transcript initiation and elongation factors from the enhancers and promoters of several oncogenes, including c-MYC, BCL2, and CDK6 (21, 25, 27). Recently, JQ1 was demonstrated to deplete the binding of BRD4, mediator, and pTEFb to the clusters of enhancers called the "super enhancers" of some oncogenes such as c-MYC, CDK6, and BCL2. This was associated with the depletion of their transcript levels (21, 27–29). JQ1 treatment also induces hexamethylene-bis-acetamide (HMBA)-inducible protein 1 (HEXIM1), which binds to cyclin T and sequesters pTEFb in an inhibitory complex that also includes the noncoding 7SK RNA (30, 31). This reduces the available kinase activity of pTEFb for phosphorylating and inducing the pause release of RNAP2, thereby contributing to the inhibition of the mRNA transcript elongation and expression of the oncogenes (21, 23, 28). Collectively, JQ1-mediated inhibitory effects on the oncogene transcription are associated with growth inhibition and apoptosis of AML cells (32–35). This has been especially noted for AML cells expressing the mutant NPM1 (NPM1c+) or mixed lineage leukemia (MLL) containing fusion oncoproteins, with or without the expression of FLT3-ITD (33–35). We also previously reported that cotreatment with JQ1 and the pan-histone deacetylase (HDAC) inhibitor (HD1) panobinostat (PS), exerts synergistic lethality against the cultured and primary AML BPCs expressing FLT3-ITD (34).

In the present studies, we demonstrate that cotreatment with JQ1 and the FLT3-TKIs ponatinib or quizartinib is synergistically lethal against human cultured or primary AML BPCs expressing FLT3-ITD. The combination concomitantly further attenuates the levels of progrowth and prosurvival proteins, for example, c-MYC, BCL2, and CDK4/6, while simultaneously inducing the levels of p21, HEXIM1, and the proapoptotic protein BIM. Finally, JQ1 alone or in combination with PS was highly active against FLT3-TKI-resistant AML cells (MOLM13-TKIR) expressing FLT3-ITD.

Materials and Methods

Reagents

(5)-JQ1 (active enantiomer, hereafter referred to as JQ1) was developed as previously described (25). I-BET151 and ABT-199 were obtained from Xcess Biologicals. PS was kindly provided by Novartis Pharmaceuticals Inc. AC220 and ponatinib were obtained from Selleck Chemicals. Chemical structures for the molecules are provided as Supplementary Fig. S1. All compounds were prepared as 10 mmol/L stocks in 100% DMSO and frozen at –20°C to –80°C in 10 μL aliquots to allow for single use, thus avoiding multiple freeze–thaw cycles that could result in compound decomposition and loss of activity. Anti–cleaved PARP, anti-c-MYC, and anti-BIM antibodies were obtained from Cell Signaling. Anti-BCL2, anti-CDK4, and anti-CDK6 antibodies were obtained from Santa Cruz Biotechnologies. Anti–p21WAF antibody was obtained from Neomarkers. Anti-HEXIM1 antibody was obtained from Bethyl Labs. Anti-β-actin antibody and lentiviral short hairpin RNAs targeting BRD4 or nontargeted short hairpin RNA (shRNA) (sh-NT) were obtained from Sigma Aldrich.

Cell culture

MOLM13 cells were obtained from the DSMZ. MV4-11 cells were obtained from ATCC. All experiments with cell lines were performed within 6 months after thawing or obtaining from ATCC or DSMZ. Cell line authentication was performed by ATCC or DSMZ. The ATCC and DSMZ utilize short tandem repeat (STR) profiling for characterization and authentication of cell lines. MOLM13-TKIR and MV4-11-TKIR cells resistant to ponatinib or quizartinib were isolated by exposing the MOLM13 and MV4-11 cells, respectively, to the continuous presence of step-
wise escalating levels of ponatinib, utilizing previously described methods (36, 37). Compared with the parental MOLM13 and MV4-11 cells, the TKIR cells were characterized for cell membrane expression of FLT3 and its intracellular signaling, as well for their the cell cycle and suspension culture growth.

Next-generation sequencing of FLT3
Equal molar pools of purified PCR amplicons corresponding to FLT3 exons 1 to 23 were prepared separately from parental MOLM13 and MOLM13-TKIR cells, to which Ion Torrent library adapters were ligated (Ion Plus Fragment Library Kit; Life Technologies, catalog no. 4471252). Libraries were amplified (Ion OneTouch 200 Solutions Kit v2; Life Technologies, catalog no. 4478319), and the template-positive Ion SphereTM particles were sequenced on an Ion 316 Chip (Life Technologies, catalog no. 446616) with 500 flows (Ion PGM 200 Sequencing Kit; Life Technologies catalog no. 4474006). A total of 1.88 million (M) and 3.15 M mapped reads were obtained for the MOLM13 and MOLM13-TKIR samples, respectively. Sequences were aligned to the above-specified targeted regions of human genome (hg)19 using ion-alignement v3.6.56201 software, and variant analysis was done with VariantCaller v3.6.6335 software using the “Somatic—Low Stringency” parameter setting.

Primary normal progenitor and AML BPCs
Primary peripheral blood and/or bone marrow aspirate AML samples were obtained and prepared for the studies below, as previously described (37, 38). Banked, delinked, and de-identified, normal or AML CD34+ bone marrow progenitor/stem cells were purified, as previously described (38). The clinical presentation of the patient and mutation status of the primary AML samples utilized in these studies is provided in Supplementary Table S1.

Assessment of apoptosis by annexin-V staining
Untreated or drug-treated cells were stained with annexin-V (Pharmingen) and TO-PRO-3 iodide and the percentages of apoptotic cells were determined by flow cytometry, as previously described (36–38). For single agent treatments, the IC50 dose was determined utilizing GraphPad Prism 5 (GraphPad Software, Inc.). The combination index (CI) for each drug combination and the evaluation of the synergistic interactions were calculated by median dose effect analyses (assuming mutual exclusivity) utilizing the commercially available software Calcsyn (Biosoft; ref. 39). CI values of less than 1.0 represent a synergistic interaction of the 2 drugs in the combination.

Assessment of percentage nonviable cells
Following designated treatments, primary AML cells were washed with 1× PBS, stained with propidium iodide (PI), and analyzed by flow cytometry, as previously described (37, 38).

shRNA to BRD4
Lentiviral shRNAs targeting BRD4 or a nontarget shRNA (sh-NT) were transduced into OCI-AML3 cells, as previously described (40). Forty-eight hours posttransduction, the cells were washed with complete media and treated with ponatinib or AC220 for 48 hours to assess apoptosis of the AML cells.

RNA isolation and quantitative polymerase chain reaction
Following the designated treatments with JQ1, total RNA was isolated from cultured and primary AML cells with a High Pure RNA Isolation Kit (Roche Diagnostics) and reverse transcribed. Quantitative real time polymerase chain reaction (PCR) analysis for the expression of c-MYC, BCL-2, HEXIM1, and p21 was performed on cDNA using TaqMan probes from Applied Biosystems (34, 40). Relative mRNA expression was normalized to the expression of GAPDH.

Cell lysis, protein quantitation, and immunoblot analyses
Untreated or drug-treated cells were centrifuged, and the cell pellets were lysed and the protein quantitation and immunoblot analyses were performed, as previously described (34, 40). Immunoblot analyses were performed at least twice. The expression levels of β-Actin served as the loading control. Representative immunoblots were subjected to densitometry analysis. Densitometry was performed using ImageQuant 5.2 (GE Healthcare).

Statistical analysis
Significant differences between values obtained in a population of AML cells treated with different experimental conditions were determined using a 2-tailed, paired t-test or a one-way ANOVA analysis within an analysis package of Microsoft Excel 2010 software or using GraphPad Prism (GraphPad Software, Inc.). P values of less than 0.05 were assigned significance.

Results
Synergistic lethal effects of JQ1 and FLT3 TKI against AML cells expressing FLT3-ITD
In a previous report and in present studies, we demonstrated that treatment with JQ1 or AC220 (quizartinib; Supplementary Fig. S1) dose-dependently exerts growth inhibitory and apoptotic effects against AML cells expressing MLL fusion proteins and FLT3-ITD (refs. 33 and 34 and Supplementary Fig. S2A and S2B). In a previous study we also demonstrated that JQ1 exerted similar lethal effects against cultured and primary AML BPCs with or without expression of FLT3-ITD (34). Taken together, based on this, in the present studies we determined the lethal activity of cotreatment with JQ1 and FLT3-TKI quizartinib or ponatinib against MOLM13 and MV4-11 cells that endogenously express FLT3-ITD. Figure 1A demonstrates that cotreatment
with quizartinib and JQ1 induces significantly more apoptosis in MV4-11 and MOLM13 cells, as compared with either agent alone ($P < 0.01$). Median dose effect and isobologram analyses demonstrated that combined therapy with JQ1 and quizartinib synergistically induced apoptosis of the 2 cell types, with combination indices less than 1.0 (Fig. 1B and Supplementary Fig. S3). Treatment with JQ1 and quizartinib, versus each drug alone, or no treatment at all, mediated greater attenuation of c-MYC, BCL2, CDK4, and CDK6 (Fig. 1C). In contrast, treatment with JQ1 did not reduce the expression levels of p-FLT3 in the cultured AML cells (Supplementary Fig. S2C and S2D). As shown in the representative immunoblots, combined treatment with JQ1 and quizartinib concomitantly induced more p21, BIM, and cleaved PARP (Fig. 1C and Supplementary Fig. S2E and S2F). Similar effects of the combined treatment were observed in MOLM13 cells (Supplementary Fig. S2G). Cotreatment with JQ1 and quizartinib also resulted in greater inhibition of the levels of p-STAT5, p-AKT, and p-ERK1/2 without significant alterations in the unphosphorylated versions of these proteins (Fig. 1D). Treatment with JQ1, but not quizartinib, also markedly induced the levels of HEXIM1, which was slightly inhibited by cotreatment with quizartinib (Fig. 1C). Fig. 2A demonstrates that cotreatment with JQ1 and ponatinib also induced...
significant more apoptosis of MV4-11 and MOLM13 cells, as compared with each agent alone (P < 0.01). In addition, combined therapy with JQ1 and ponatinib was also synergistically lethal against MV4-11 and MOLM13 cells, showing combination indices of less than 1.0 (Fig. 2B and Supplementary Fig. S3). Synergistic apoptosis of the 2 cell types was also observed, following treatment with I-BET151 and ponatinib (Supplementary Fig. S4A and S4B). Treatment with JQ1 and ponatinib also inhibited the expression of c-MYC, BCL2, and CDK4/6 more than the treatment with each drug alone, while simultaneously inducing more p21, BIM, and cleaved PARP (Fig. 2C and Supplementary Fig. S2F). Cotreatment with JQ1 and ponatinib also mediated greater attenuation of p-AKT and p-ERK1/2 but not of AKT and ERK1/2 in the cells expressing FLT3-ITD (Fig. 2D). We next determined whether specific depletion of BRD4 by shRNA would phenocopy the effects of JQ1 in increasing the anti-AML...
activity of FLT3-TKI. Figure 2E demonstrates that, as compared with treatment with the nontargeted shRNA, treatment with BRD4 shRNA reduced the mRNA levels of BRD4, while simultaneously increasing the mRNA levels of p21. We have previously reported that shRNA-mediated knockdown of BRD4 also results in depletion of the mRNA levels of BCL2 and CDK6 (34). In the cells transduced with BRD4 shRNA, compared with cells transduced with the nontargeted shRNA, treatment with quizartinib (AC220) or ponatinib induced significantly more apoptosis of MOLM13 cells \( (P < 0.05; \text{Fig. 2F}) \).

JQ1 and FLT3-TKI synergistically induce apoptosis of primary AML BPCs expressing FLT3-ITD

We next compared the lethal effects of JQ1 and ponatinib or quizartinib against samples of patient-derived, CD34\(^+\) primary AML progenitor cells expressing mutant FLT3-ITD or coexpressing NPM1c\(^+\) and FLT3-ITD. Figure 3A demonstrates that, although, the loss of viability in the primary AML progenitor cells was lower than in the cultured AML cell types, exposure to 250 nmol/L JQ1 exerted greater lethal effects than exposure to 100 nmol/L of ponatinib. Cotreatment with JQ1 and ponatinib was more lethal than each agent alone against the primary
AML BPCs (Fig. 3A). Importantly, combined treatment with JQ1 and ponatinib exerted significantly greater lethality against the primary AML versus normal progenitor cells (Fig. 3A). In addition, cotreatment with JQ1 and ponatinib or quizartinib was synergistically lethal against primary AML BPCs, showing combination indices of less than 1.0 (Fig. 3B and C). This synergy was observed whether FLT3-ITD was expressed alone or with additional mutation such as NPM1c (Supplementary Fig. S5A and S5B). Cotreatment with JQ1 and ponatinib or quizartinib was synergistically lethal, regardless of the coexpression of FLT3-ITD with or without NPM1c (Supplementary Fig. S5C and data not shown). As was observed in the cultured AML cell types, treatment with JQ1 and ponatinib or quizartinib versus each drug alone, or no treatment at all, mediated greater attenuation of p-RNAp2, c-MYC, BCL2, CDK4, and CDK6 in the primary AML BPCs (Fig. 3D and 3E). Here, also, cotreatment with JQ1 and ponatinib or quizartinib mediated greater attenuation of p-akt, p-ERK1/2, and p-STAT5 but not of AKT, ERK1/2, STAT5, or p-FRT (Fig. 3D and 3E and Supplementary Fig. S6). As compared with each agent alone, combined treatment with JQ1 and ponatinib or quizartinib simultaneously induced more p21, BIM, and cleaved PARP in the primary AML progenitor cells (Fig. 3D and 3E and Supplementary Fig. S6C).

**JQ1 induces apoptosis of cells expressing mutations in the TKD of FLT3**

Because an important secondary mechanism of resistance to therapy with FLT3-TKIs in AML is the emergence of mutations in the activation loop of FLT3-TKD2 (FLT3-D835V/Y/F) and in the FLT3-TKD1 (FLT3-F691L), we next determined the activity of JQ1 against BaF3 cells stably expressing FLT3-ITD or FLT3-ITD along with either FLT3-F691L or with FLT3D835V (4). As previously reported, unlike the parental BaF3 cells, FLT3-ITD expressing cells are able to grow in a culture medium devoid of IL3 (3, 4). As shown in Fig. 4A, treatment with JQ1 dose-dependently induced apoptosis of not only BaF3 cells expressing FLT3-ITD but also of those coexpressing FLT3-ITD along with either F691L or with D835V substitution. The IC50 values for JQ1 for inducing apoptosis in the 3 cell types were: FLT3-ITD, 697 nmol/L; FLT3-ITD-F691L, 1,588 nmol/L; and FLT3-ITD-D835V, 909 nmol/L. JQ1-induced apoptosis in the BaF3 cells expressing FLT3-ITD, FLT3-ITD-F691L, and FLT3-ITD-D835V was associated with JQ1-mediated attenuation of c-MYC, but induction of HEXIM1 and BIM (the extra-long, long, and short isoforms; Fig. 4B). Thus, JQ1 retains effectiveness against cells expressing the clinically encountered mutations in FLT3 that confer resistance to FLT3-TKI.

**Superior activity of JQ1 against the FLT3-ITD expressing human AML cells highly resistant to FLT3-TKI**

To determine whether JQ1 would also be active against FLT3-ITD expressing, FLT-TKI–resistant human AML cells expressing the clinically encountered mutations in isoforms; Fig. 4B). Thus, JQ1 retains effectiveness against cells expressing the clinically encountered mutations in FLT3 that confer resistance to FLT3-TKI.

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that has been previously discovered and reported following in vitro or in vivo exposure toFLT3-TKIs, including quizartinib or ponatinib (2, 4, 6, 14). However, we did detect a very low allelic frequency mutation in FLT3 represented by the A680V substitution in MOLM13-TKIR cells (Fig. 5D), which has not been previously shown to confer in vivo resistance to FLT3-TKIs. Notably, compared with MOLM13, MOLM13-TKIR cells also expressed higher mRNA and protein levels of p21 and HEXIM1 (Fig. 6A–C). In addition, higher protein levels of BRD4, c-MYC, BCL2, Bcl-xL, CDK6, BIM, p-AKT, and AKT were observed in MOLM13-TKIR versus MOLM13 cells (Fig. 6C). Although markedly inducing the levels of p21, HEXIM1, BRD4, BIM, and cleaved PARP, treatment with JQ1 dose-dependently attenuated the levels of c-MYC, BCL2, Bcl-xL, CDK6, p-AKT, and AKT in MOLM13-TKIR and MOLM13 cells (Fig. 6C). Importantly, MOLM13-TKIR cells were significantly more sensitive than the parental MOLM13 cells to JQ1-induced apoptosis, for example, exposure to 100 or 1000 nmol/L of JQ1 induced significantly more apoptosis of MOLM13-TKIR versus MOLM13 cells (P < 0.01; Fig. 6D). The IC50 values for JQ1-induced apoptosis in MOLM13-TKIR versus MOLM13 cells were 269.4 nmol/L versus 1480 nmol/L, respectively (Supplementary Table 2). The observed collateral sensitivity of MOLM13-TKIR cells to JQ1 was associated with greater induction of p21, HEXIM1, BIM, and cleaved PARP by JQ1 treatment in MOLM13-TKIR versus MOLM13 cells. We next determined whether the combination of JQ1 with AC220 would exert greater antileukemia activity against the MOLM13-TKIR cells. In contrast to the synergistic anti-leukemia activity of JQ1 and AC220 in MOLM13 cells, the combination of JQ1 and AC220 was no more effective than treatment with JQ1 alone in the MOLM13-TKIR cells (Supplementary Fig. S7). We also determined the effect of the knockdown of BRD4 by shRNA on the induction of apoptosis in MOLM13-TKIR versus MOLM13 cells. As shown in the
Supplementary Fig. S8, knockdown of BRD4 (approximately 80%) did not significantly induce apoptosis in either MOLM13-TKIR or MOLM13 cells (approximately 10% in each cell type). However, the knockdown of BRD4 further sensitized MOLM13-TKIR more than MOLM13 cells to treatment with JQ1 (Supplementary Fig. S8B). We also determined the mechanistic role of BCL2 overexpression on the sensitivity of MOLM13-TKIR versus MOLM13 cells to apoptosis. We treated MOLM13-TKIR and MOLM13 cells with the BCL2-specific antagonist ABT-199. As shown in Supplementary Fig. S8C, MOLM13-TKIR cells were not more sensitive than MOLM13 cells to treatment with ABT-199.

Cotreatment with JQ1 and PS synergistically induces apoptosis of AML cells resistant to FLT3-TKI

As shown in Fig. 7A, as compared with the parental MOLM13 cells, MOLM13-TKIR cells displayed higher levels of the class I and class IIb HDACs HDAC1, HDAC2, HDAC3, and HDAC6. In addition, MOLM13-TKIR cells expressed higher levels of the polycomb repressive complex 2 (PRC2) proteins EZH2 and SUZ12 (Fig. 7A). We had previously reported that PS treatment depletes EZH2 and SUZ12 in AML cells (38). As shown in Supplementary Table S2, PS and vorinostat (VS) were as active in inducing apoptosis in MOLM13-TKIR as in MOLM13 cells. In addition, Fig. 7A also demonstrates that treatment with JQ1 alone dose-dependently, but modestly, attenuated the levels of HDAC1, HDAC2, HDAC3, EZH2, and SUZ12, without affecting the levels of HDAC6. We had also previously reported that combined therapy with JQ1 and PS exerts synergistic lethality against cultured and primary human AML BPCs, including those expressing FLT3-ITD such as MOLM13 cells (34). Collectively, based on this, in the present studies we determined the activity of JQ1 and PS against
FLT3-TKI–resistant MOLM13-TKIR cells. As compared with each agent alone, combined treatment with JQ1 and PS induced significantly more apoptosis of MOLM13-TKIR cells with and without resistance to FLT3-TKI. Figure 7B demonstrates that cotreatment with JQ1 and PS synergistically induced apoptosis of MOLM13-TKIR cells, with combination indices of less than 1.0 by the median dose effect and isobologram analysis. CI values less than 1.0 indicate a synergistic interaction of the 2 agents in the combination. Following this, the percentage of annexin V–positive, apoptotic cells was determined by flow cytometry. Columns, mean of 2 experiments; bars, SD. *Apoptosis values significantly greater in the combination, compared with treatment with either agent alone (P < 0.05). Cotreatment with JQ1 and PS synergistically induced apoptosis of MOLM13-TKIR cells with and without resistance to FLT3-TKI. As compared with each agent alone, combined treatment with JQ1 and PS induced significantly more apoptosis of MOLM13-TKIR cells with and without resistance to FLT3-TKI. 

Discussion

The FLT3-TKIs quizartinib and ponatinib are potent type II FLT3 antagonists that bind and inhibit the inactive conformation of FLT3-TK, where the activation loop is in the DFG "out" position (4, 6). Although they inhibit growth and induce differentiation and apoptosis, as well as induce complete remission in patients with AML, following treatment with the FLT3-TKI, relapse, because of residual AML in which FLT3-ITD activity is persistent, is commonly encountered (2, 4, 6, 14). Several mechanisms of primary resistance to FLT3-TKI have been described in AML BPCs expressing FLT3-ITD. These include the protective effects of stromal niche cells (41), increased levels and activity of FLT3 ligand on FLT3 TK (15, 17), trans-
phosphorylation and increased activity of FLT3 by other kinases such as PIM-1 and SYK (42, 43), and the collateral dependency on other important progrowth and prosurvival signaling mechanisms (5, 6, 12, 13). Other studies have focused on developing selective and more potent FLT3-TKIs to overcome primary resistance to the first and second generation FLT3-TKIs already in the clinic (44, 45). Here, we document for the first time that cotreatment with the BET protein antagonist (BA) JQ1 and FLT3 inhibitor quizartinib or ponatinib synergistically induced apoptosis of not only cultured but also of the primary AML BPCs expressing FLT3-ITD. By disrupting BRD4 activity as a chromatin “reader” protein and by inducing HEXIM1, JQ1 in combination with quizartinib or ponatinib depleted the levels of the progrowth and prosurvival oncogenes in AML, including c-MYC, BCL2, and CDK4/6. The synergistic anti-AML activity of cotreatment with JQ1 and the TKIs could also be partly explained by the concomitant marked depletion of p-STAT5, p-AKT, and p-ERK1/2, but significant induction of p21 and BIM, collectively known to undermine the growth and survival of AML cells expressing FLT3-ITD (2, 5, 6, 12, 13). These findings are consistent with a recent report in which JQ1 was shown to inhibit p-STAT3 and be active against myeloid malignancies (46). It is noteworthy that whereas the genetic knockdown of BRD4 enhanced the anti-AML lethality of quizartinib or ponatinib, this effect was less pronounced than the effect of BRD4 inhibition by cotreatment with JQ1, which mediates its growth inhibitory and lethal effects by inhibiting BET proteins in addition to BRD4. Collectively, these data highlight the promise of including a BET protein antagonist in combination with quizartinib or ponatinib for the treatment of high-risk AML expressing FLT3-ITD.

Findings presented here also for the first time demonstrate that JQ1 exerts significant lethal activity against the cultured mouse BaF3 cells expressing FLT3-ITD alone or with the FLT3-TKD mutations F691L or D835V. This is most likely because JQ1 is known to inhibit the “transcriptome” responsible for the growth and survival that is activated downstream of the “gain-of-function” mutations in FLT3 and the signaling downstream of it. Although the compound mutations FLT3-ITD-F691L and FLT3-ITD-D835V confer resistance to quizartinib and ponatinib, respectively, they likely continue to confer dependency on the BET protein-regulated “transcriptome” for growth and survival and, hence, remain susceptible to the inhibitory effects of JQ1. A novel class I TKI inhibitor, crenolanib, which is capable of inhibiting the active “DFG in” conformation of FLT3, demonstrated activity against FLT3 containing an ITD and/or D835- or F691-activating mutations (47). It would be important to determine the synergistic effects of JQ1 and crenolanib against AML expressing FLT3-ITD and/or D835- or F691-activating mutations. Because of the lack of availability of adequate samples of primary AML BPCs expressing FLT3-D835V or FLT3-F691L, or compounds mutations including FLT3-ITD with F691L or D835V, we determined the activity of JQ1 against the cultured FLT3-TKI–resistant MOLM13-TKIR cells. These cells lacked any FLT3 mutation that has been documented in the clinic to confer resistance to FLT3-TKI. Instead, they exhibited high levels of resistance to quizartinib and ponatinib associated with deregulated expressions of a number of proteins that confer growth and survival advantage in AML cells.

Notably, as compared with the parental MOLM13, MOLM13-TKIR cells showed markedly elevated levels of EZH2 and SUZ12, suggesting the possibility that MOLM13-TKIR cells may be relatively more dependent on PRC2-regulated genes, hence more susceptible to inhibition of EZH2 and SUZ12. Our data demonstrate that treatment with JQ1 also attenuated the levels of EZH2 and SUZ12 (Fig. 7A), which may further contribute to the greater susceptibility of MOLM13-TKIR versus MOLM13 cells to the lethal effects of JQ1. It should be noted that deregulated expressions of the epigenetic modifier proteins shown in MOLM13-TKIR cells have yet to be evaluated in patient-derived FLT3-TKIR–resistant primary AML cells expressing FLT3-ITD but lacking the expression of point substitutions in FLT3 known to confer resistance to FLT3-TKIs.

In a previous report by us it was demonstrated that cotreatment with PS and JQ1 exerted synergistic lethality against AML cells coexpressing MLL fusion oncprotein, or mutant NPM1c–, and FLT3-ITD (34). This was partially attributed to PS-induced hyperacetylation of histone proteins and recruitment of BRD4 to active gene promoters, thereby increasing the sensitivity of the AML cells to JQ1 (34). Based on this, and the demonstration here that JQ1 alone is considerably more active against the FLT3-TKI–resistant MOLM13-TKIR cells versus MOLM13 cells, we determined the lethal effects of cotreatment with PS and JQ1 against MOLM13-TKIR cells. Findings presented here confirm that cotreatment with PS and JQ1 is synergistically lethal against MOLM13-TKIR cells. Similar effect of the combination was noted against FLT3-TKIR–resistant MV4-11-TKIR cells. Collectively, these findings indicate that although JQ1 alone is active against AML expressing FLT3-ITD and Ba/F3 cells with ectopic expression of FLT3-ITD with or without concurrent FLT3-TKD mutations, cotreatment with PS and JQ1 is synergistically lethal against AML expressing FLT3-ITD alone or with FLT3-TKD mutations. Recently, the AML stem/progenitor cells (LSC) expressing DNMT3A mutation were demonstrated to exhibit repopulation potential and therapeutic resistance to chemotherapy (48). Targeting LSCs is an
important goal in achieving deeper remissions and overcoming treatment refractoriness in the minimal residual AML, because LSC gene expression program confers a poor prognosis and influences the clinical outcome in AML (49, 50). Previous reports have also clearly demonstrated that the synergistic combination of PS and JQ1 is lethal against AML BPCs expressing DNMT3A, such as OCI-AML3 cells, as well as against immunophenotypically defined CD34+, CD38−, and lineage negative primary AML stem/progenitor cells that exhibit in vivo leukemia initiating potential (34, 48). Taken together with these reported observations, our findings presented here underscore the potential of combined therapy with FLT3-TKI or PS and JQ1 to eradicate AML stem cells and clonally derived AML BPCs that carry FLT3-TKD with or without FLT3-TKD mutations and documented to be responsible for therapeutic resistance in AML.

Disclosure of Potential Conflicts of Interest
S. Sharma has received speakers’ bureau honoraria from Beta Cat Pharmaceuticals, Salarus Pharmaceuticals, and ConverGene. J.E. Bradner has ownership interest (including patents) in and is a consultant/advisory board member for Tensoha Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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