PFI-1, a Highly Selective Protein Interaction Inhibitor, Targeting BET Bromodomains

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Abstract

Bromo and extra terminal (BET) proteins (BRD2, BRD3, BRD4, and BRD7) are transcriptional regulators required for efficient expression of several growth promoting and antiapoptotic genes as well as for cell-cycle progression. BET proteins are recruited on transcriptionally active chromatin via their two N-terminal bromodomains (BRD), a protein interaction module that specifically recognizes acetylated lysine residues in histones H3 and H4. Inhibition of the BET–histone interaction results in transcriptional downregulation of a number of oncogenes, providing a novel pharmacologic strategy for the treatment of cancer. Here, we present a potent and highly selective dihydroquinazoline-2-one inhibitor, PFI-1, which efficiently blocks the interaction of BET BRDs with acetylated histone tails. Co-crytal structures showed that PFI-1 acts as an acetyl-lysine (Kac) mimetic inhibitor efficiently occupying the Kac binding site in BRD4 and BRD2. PFI-1 has antiproliferative effects on leukemia cell lines and efficiently abrogates their clonogenic growth. Exposure of sensitive cell lines with PFI-1 results in G1 cell-cycle arrest, downregulation of MYC expression, as well as induction of apoptosis and induces differentiation of primary leukemic blasts. Intriguingly, cells exposed to PFI-1 showed significant downregulation of Aurora B kinase, thus attenuating phosphorylation of the Aurora substrate H3S10, providing an alternative strategy for the specific inhibition of this well-established oncology target. Cancer Res; 73(11); 3336–46. © 2013 AACR.

Introduction

Bromodomains (BRD) are protein interaction modules that specifically recognize ε-N-acetylated lysine residues (1, 2). BRDs are common interaction modules in nuclear proteins that regulate gene transcription and chromatin organization and play a key function recruiting these protein complexes to acetylated chromatin. Dysfunction of BET-containing proteins has been linked to the development of diverse diseases in particular to the development of cancer (3).

Bromodomains are highly sequence diverse but they share a conserved fold that comprises a left-handed bundle of 4 alpha helices (αZ, αA, αB, αC; ref. 4). The acetyl-lysine side chain is typically anchored by a hydrogen bond to a conserved asparagine residue and water-mediated interactions with a conserved tyrosine (2, 5). Crystal structures of bromo and extra terminal (BET) complexes with di-acetylated histone 4 tail peptides showed that the first BRDs of BRD4 and BRD7 may accommodate 2 acetyl-lysines in a single binding site (2, 6).

The BET family of BRD proteins comprises 4 members in mammals (BRD2, BRD3, BRD4, and BRD7) each containing 2 conserved N-terminal BRDs. BET proteins play critical roles in cellular proliferation and cell-cycle progression (7). Genetic rearrangement of the BRD4 and BRD3 locus in which in-frame chimeric proteins of the N-terminal BRDs of BRD4 or BRD3 with the protein NUT (nuclear protein in testis) give rise to the development of NUT midline carcinoma (NMC), an incurable uniformly fatal subtype of squamous carcinoma (8). BRD4 has been shown to be critical for survival of a number of diverse tumors due to its function promoting transcription of growth promoting and antiapoptotic genes (9) which prompted the development of potent and selective protein interaction inhibitors targeting BET BRDs. The potent pan-BET inhibitors (+)-JQ1 and GSK1210151A (I-BET151) have shown significant antitumor activity in murine models of NMC (10).
myeloma (11), acute myeloid leukemia (AML; ref. 12), and mixed lineage leukemia (MLL; ref. 13). Genetic knockdown by RNAi or exposure of cells with BET inhibitors resulted in a significant transcriptional downregulation of MYC (11).

In contrast to most transcriptional regulators that dissociate from chromatin during mitosis, BRD4 preferentially associates with mitotic chromosomes (14) "bookmarking" G2, and growth-associated genes for efficient postmitotic transcription, providing a mechanism for transcriptional memory during cell division (15). BRD4 knockdown in primary human keratinocytes by RNAi results in severe cytokinesis defects and down-regulation of Aurora B expression. Aurora kinases (A, B, and C) are essential for mitotic entry and progression (16). The A and B isoforms play distinct roles during mitosis: Aurora A is required for mitotic spindle assembly during pro and metaphase, whereas Aurora B is part of the mitotic passenger complex mediating chromosome segregation by ensuring proper biorientation of sister chromatids during metaphase and anaphase (17, 18). Both Aurora isoforms are highly expressed in cancer, augment Ras-induced transformation, and have therefore emerged as attractive therapeutic targets (19, 20). Interestingly, transcription of Aurora A and B is strongly upregulated by the BRD4 target gene Myc, whereas, in turn, Aurora kinases also have a critical function in regulating c-Myc turnover by regulating protein stability, suggesting that activity of these growth promoting proteins is tightly regulated by a feedback loop (21).

Here, we describe a novel highly potent inhibitor PFI-1 that selectively targets BET BRDs. PFI-1 binds to BET BRDs with low nanomole potency and is chemically distinct from previously reported BET inhibitors. Exposure of leukemia cells to PFI-1 results in induction of caspase-dependent apoptosis, differentiation, and in downregulation of the Aurora B kinase. This highly selective chemical probe provides a versatile tool for further validation of BRD4 in cancer and other diseases and suggests synergism between 2 major oncogenes, c-Myc and Aurora B that can be simultaneously targeted by selective BET inhibition.

Materials and Methods

BRD2, BRD3, BRD4, BRD5, and CREBBP BRDs were cloned, expressed, and purified as previously described (10). For biotin labeling, CREBBP (R1081-G1198) and BRD2 (K71-N195) were subcloned into pNIC-BIO1 (Gene Bank: EF198106) and expressed in BL21 (DE3)-R3-BirA. d-Biotine was dissolved into 10 mmol/L bicine pH 8.3 and added to the culture at 500 μmol/L final. Biotinylated protein was immobilized on Super Streptavidin Biosensors using 50 mmol/L HEPES pH 7.4, 100 mmol/L NaCl, and 0.01% Tween.

Isothermal titration calorimetry (ITC), temperature shift assays, and fluorescence recovery after photobleaching were carried out as previously described (22). All cell lines were obtained from American Type Culture Collection and were cultured in RPMI-1640 medium (Sigma) containing 10% FBS. In vitro cytotoxicity assays were conducted in triplicate using either Cell-Titer-Glo reagent (Promega) or WST-1 (Roche) according to instructions provided by the vendor.

CD34+ human hematopoietic stem cells, obtained from peripheral blood of healthy patients, were plated in methylcellulose (StemAlpha) supplemented with human cytokines (H4535, Stem Cell Technologies) at a cell dose of 1 × 10⁴ per plate.

B-ALL primary graph

NOD/Shi-scid/IL-2Rγnull (NOG) mice were engrafted with B-acute lymphoblastic leukemia (B-ALL) primary blasts t(1, 19)+ using a sample obtained from a 9-year-old child with high-risk B-ALL. The engraftment process was monitored by staining peripheral blood samples for human CD45 and mouse CD45. At 5 weeks, hCD45 was at least 1% and a treatment cycle was initiated by injection of 50 mg/kg JQ1 4 times per week into the peritoneum (IP) for 3 weeks. Bone marrow was extracted from the femur of JQ1 and vehicle [dimethyl sulfoxide (DMSO)]–treated mice, fixed in 10% formalin, and mounted in paraffin blocks.

Fixation, immunostaining, and confocal microscopy

Approximately 5 × 10⁴ cells were used for each cytospot. Cells were centrifuged at 500 rpm for 5 minutes using a Cytospin 3 SHANDON cytocentrifuge. Fixation of the cells was carried out with 4% paraformaldehyde solution in PBS. Incubation was conducted for 16 hours at 4°C in 0.3% Triton X-100, 0.5% BSA, and the indicated primary antibody. After washing, the cells were incubated with the secondary antibody for 2 hours. The cells were washed and a coverslip was placed on top by adding a droplet of mounting solution (Clear-Mount, Invitrogen). Confocal fluorescence images were obtained by a LSM710 microscope (Zeiss).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were conducted using the EZ-Chip Kit by Millpore according to the manufacturer’s protocol. Results were quantified both by semiquantitative and quantitative PCR, conducted using Terra qPCR Direct SYBR Premix (Clonetech) on an ABI 7900HT.

Results

In a screen for putative acetyl-lysine mimetic compounds, we identified the simple fragment 6-bromo-3-methyl-3,4-dihydroquinazoline-2-one as a BRD4 and CREBBP BRD inhibitor. This inhibitor displaced tetra-acetylated histone 4 peptides in ALPHA (amplified luminescent proximity homogeneous assay) screen assays with an IC₅₀ value of approximately 50 μmol/L for the first domain of BRD4 [BRD4(1)]. Expansion of this scaffold resulted in a series of highly potent and specific benzenesulfonamide-quinazolin-2-one BET inhibitors. PFI-1 was selected as one of the most potent and selective compounds of about 300 inhibitors profiled in ALPHA screen assays (Fig. 1A). The inhibitor was synthesized as outlined in Supplementary Fig. S1. Structure–activity relationship of this class of compounds will be described elsewhere (23).

PFI-1 is a potent and selective BET bromodomain inhibitor

ALPHA screen assays have shown that PFI-1 displaces histone 4 peptide acetylated at lysines K5, K8, K12, and K16 (H4K5acK8acK12acK16ac) with a potency (IC₅₀) of 220 nmol/L.
for BRD4(1) and 98 nmol/L for BRD2(2). The tight interaction of PFI-1 with BET acetyl-lysine-binding sites was confirmed by ITC, which revealed dissociation constants ($K_D$) of PFI-1 of 47.4 ± 2.5 nmol/L [BRD4(1)] and 194.9 ± 6 nmol/L [BRD4(2)] for the 2 BRDs present in BRD4. ITC titrations against BRDs of the BET family showed that PFI-1 bound with similar affinities (Fig. 1B and Supplementary Table S1). Binding of PFI-1 was strongly driven by large negative binding enthalpy change, suggesting that polar interactions of PFI-1 with BET BRDs are highly favorable. Interestingly, binding enthalpies ($ΔH$) were between −4 and −7 kcal/mol larger for N-terminal BET BRDs. However, this large difference in favorable binding enthalpy was almost completely compensated by unfavorable changes in binding entropy ($ΔS$) resulting in similar binding constants. The largest difference in binding affinity (about 4 fold) was observed for the 2 BRDs of BRD4.

Comprehensive screening of PFI-1 against 42 human BRDs using temperature shift assays (10, 24) have shown high specificity of PFI-1 for the BET family of BRDs (Fig. 1C and Supplementary Table S1). Binding of PFI-1 was strongly driven by large negative binding enthalpy change, suggesting that polar interactions of PFI-1 with BET BRDs are highly favorable. Interestingly, binding enthalpies ($ΔH$) were between −4 and −7 kcal/mol larger for N-terminal BET BRDs. However, this large difference in favorable binding enthalpy was almost completely compensated by unfavorable changes in binding entropy ($ΔS$) resulting in similar binding constants. The largest difference in binding affinity (about 4 fold) was observed for the 2 BRDs of BRD4.

Figure 1. Potency and selectivity of PFI-1. A, chemical structure of PFI-1. B, selectivity screening data of PFI-1 using temperature shift assays. Screened targets are highlighted in bold. Temperature shifts are indicated by red filled circles with increasing radii for higher $T_m$ values as indicated in the figure. C, isothermal titration data measured on BRD4(1; black) and BRD4(2; red). Shown are heat effects for each injection and the normalized binding isotherms (insert) including the fitted function (solid line). D, ALPHA-screen data measured using isolated BRDs of BRD4(1; red triangle) BRD4(2; blue triangle) as well as the BRD of CREBBP (green circle). Fitted functions are show as solid lines.

PFI-1 binds to the acetyl-lysine–binding site of BET bromodomains

Cocrystallization of PFI-1 with BRD4(1) revealed the binding mode of this highly specific BET BRD inhibitor. Crystals of the BRD4(1)/PFI-1 complex diffracted to high (1.52 Å) resolution providing a detailed view of the interactions formed by this histone acetyl transferases CBP/EP300. The interaction with CBP was too weak for determination of an accurate $IC_{50}$ value using ALPHA screen assays. However, we estimated an affinity of PFI-1 for CBP of approximately 11 μmol/L using Bio-Layer Interferometry (BLI; Supplementary Fig. 1B; ref. 25), suggesting more than 300-fold selectivity over the 2 BRDs that showed the largest $ΔT_m$ shift outside the BET BRD family. In comparison, BLI determined an affinity of 111 nmol/L for BRD2(1) in agreement with ITC and ALPHA screen data. We also assessed activity of PFI-1 outside the BET family. Screening against 38 protein kinases revealed no significant inhibitory activity of PFI-1. Similarly, no significant activity was observed screening PFI-1 against 40 human kinases and 14 human membrane receptors (Supplementary Tables S3 and S4).
inhibitor with the BRD acetyl-lysine–binding site. Details on data collection and refinement are summarized in Supplementary Table S5. The overall structure of the PFI-1 in complex with BRD4(1) revealed the typical helical fold and structural elements of BRDs (Fig. 2A) and comparison with the apos-structure (PDB-ID:2OSS) showed only minor structural rearrangements in the ZA-loop regions. The inhibitor and coordinating residues and water molecules were well defined by electron density (Fig. 2B). PFI-1 showed an extraordinary shape complementarity with the Kac binding site. The quina-zolinone carbonyl and nitrogen acted as a hydrogen bond donor/acceptor pair that interacted with the conserved asparagine N140. The quinazolinone carbonyl also formed a second water-mediated hydrogen bond with the conserved residue Y97. The network of 5 tightly bound water molecules that are typically found at the base of BRD acetyl-lysine–binding pocket was also conserved in the BRD4(1) PFI-1 complex. Comparison to BRD4(1) structures of diacetylated peptide complexes (2) confirmed the acetyl-lysine mimetic binding mode of PFI-1 (Fig. 2C). The carbonyl moieties of the quinazolinone and the acetyl-lysine are in similar position and mediate the same interactions in superimposition of both BRD4(1) ligand complexes. Superimposition with the BRD4(1)/(+)-JQ1 complex revealed a largely diverse binding mode. However, both complexes form an acetyl-lysine mimetic hydrogen bond to N140 as well as aromatic stacking and hydrophobic interactions with W81 and the shelf region of the acetyl-lysine–binding site.

ITC data showed a significantly smaller binding enthalpy change to second BRDs of BET family members and we were interested if the cocystal structure of PFI-1 with a second BRD would explain these striking differences in the observed binding thermodynamics. Comparison of the structure of the PFI-1 complex of BRD4(1) and BRD2(2) showed that polar interaction of the inhibitor with BRD2(2) are less optimal (Fig. 2D and E). The hydrogen bond between the quinazolinone NH with the conserved asparagine residue [N140 in BRD4(1) and N429 in BRD2(2)] is less favorable (distance of 3.3 Å) in the case of BRD2 (2) when compared with BRD4(1; distance of 2.9 Å). In addition, the water-mediated hydrogen bond between the sulfonamide NH function and the ZA loop backbone carbonyl observed in
the BRD4(1) complex (3.2 Å) is further away from the bridging water molecule which was, however, still observed in the BRD2 (2) cocrystal structure (3.8 Å). Interestingly, in contrast to (+)-JQ1 BRD complexes, H433 reoriented and flipped into the acetyl-lysine–binding site to interact with the methoxybenzyl moiety of PFI-1. This large structural rearrangement may provide an explanation for the large difference in ΔS upon binding of PFI-1 to the first and second BRDs. H433 is conserved in all second BRDs, whereas in N-terminal BET BRDs, this residue is always an aspartate [D144 in BRD4(1)]. This difference in Kac site composition may be explored for the development of inhibitors that preferentially recognize either the first or second BRD.

**PFI-1 displaces BRD4 from chromatin**

To establish whether PFI-1 dissociates full-length BRD4 from acetylated chromatin in cells, we developed fluorescence recovery after photo-bleaching (FRAP) experiments in human osteosarcoma cells (U2OS) transfected with GFP–BRD4. The use of FRAP in assessing the diffusion of BRD-containing proteins tagged with fluorescent fusion partners and therefore providing evidence of the level of chromatin association has been previously established (22), and we have successfully used this method to show the on-target effect of the BET inhibitor JQ1 in BRD4-dependent NMC cell lines (10). Cells treated with 1 and 5 μmol/L PFI-1 showed significantly faster fluorescent recovery times when compared with cells that have not been exposed to this inhibitor, suggesting that full-length BRD4 was displaced from chromatin in PFI-1–treated cells (Fig. 3). Differences in fluorescent recovery time of PFI-1–treated cells were comparable with effects observed for the biochemically active (+)-JQ1 stereoisomer.

**PFI-1 inhibits proliferation of a subset of leukemic cells**

We used a luminescent ATP-based cytotoxicity assay to examine the sensitivity of a panel of established leukemia cell lines to PFI-1 in a dose-dependent manner (Table 1). In agreement with earlier studies, we found that cell lines carrying oncogenic rearrangements in the MLL locus (26) such as MV4;11 (MLL-AF4), NOMO-1 (MLL-AF9), SEMKH2 (MLL-AF4) RS4;11 (MLL-AF4), or THP-1 (MLL-AF9) were highly sensitive to BET inhibition (12, 13, 27). Comparable activity was also observed for the AML1–ETO fusion oncogenes bearing AML cell line Kasumi. Significantly less sensitive (5–10 μmol/L) was the ALL-derived cell line KOCL-45 despite its MLL–AF4 rearrangement and the human histiocytic lymphoma cell line U937. No significant activity was detected for K-562 [BCR-ABL–positive blast crisis chronic myelogenous leukemia (CML)] and PL-21 (AML) showing that the growth inhibition observed in certain leukemia cell lines is not due to a general cytotoxicity of PFI-1. However, the activity of PFI-1 on cell proliferation of leukemia cell lines was between 5- and

![Figure 3. FRAP data showing dissociation of GFP-BRD4 from chromatin. A, nuclei of PFI-1–treated (top) and untreated (bottom) cells. The bleached area is indicated by a read polygon. B, time dependence of fluorescent recovery in the bleached area for DMSO-treated, (+)-JQ1–treated, and PFI-1 (1, 5 μmol/L)-treated cells. C, half times of fluorescence recovery of DMSO, (+)-JQ1, and PFI-1 (1, 5 μmol/L)-treated cells. The data shown represent the average values of 20 experiments.](image-url)
We also investigated the efficiency of PFI-1 to suppress the clonogenic growth of leukemic cells in methylcellulose. In agreement with the cytotoxicity assays in liquid culture, we observed strong ablation of clonogenic growth in the PFI-1–sensitive cell lines MV4:11 and THP-1 (Fig. 4). However, clonogenic growth of human CD34–positive human hematopoietic stem cells from 2 different healthy donors was also significantly impaired. Interestingly, colony formation of the PFI-1 insensitive cell line K562 was not affected, but exposure to PFI-1 did significantly reduce cell numbers, suggesting that growth is still compromised in this cell line. This effect was also observed using (+)-JQ1 (Fig. 4C–E). The sensitivity of cell lines to BET-dependent growth inhibition was independent of BRD4 mRNA expression levels. We conducted quantitative real-time PCR (qRT-PCR) experiments quantifying the levels of the 2 main BRD4 isoforms [long (GI:7657218)] and short (GI:19718731)]. We found comparable levels of the short BRD4 isoforms in all cell lines. mRNA expression levels of the long isoform were particularly high in K-562 and THP-1 cells (Supplementary Fig. S3).

PFI-1 induces cell-cycle arrest and apoptosis in sensitive cell lines

Annexin V staining combined with FACS analysis established strong induction of apoptosis by BRD4 knockdown or chemical inhibition of BET proteins (12, 13, 27). Here, we were interested to characterize the mechanism of PFI-1–induced apoptosis using Western blot analysis of proteins known to play key roles in apoptosis. In the BET inhibitor–sensitive cell line MV4:11, we observed strong induction of PARP1 and procaspase 7 cleavage after 24-hour exposure with PFI-1, whereas protein levels as well as the phosphorylation state of the proapoptotic protein BAD were unaffected. In contrast, neither PARP1 nor procaspase 7 cleavage was observed in the PFI-1 insensitive cell line K-562 (Fig. 5). In agreement with earlier studies using (+)-JQ1, we also detected significantly lower c-Myc protein levels in PFI-1–treated MV4:11 cells, but only a minor reduction in c-Myc levels was observed in K-562 cells (12, 27). This analysis shows that the canonical caspase-induced apoptotic pathway is activated in cell lines that are sensitive to BET BRD inhibition by low molecular weight inhibitors. As predicted from RNAi knock down studies and studies on (+)-JQ1 (10) that have shown a key role of BRD4 in cell-cycle progression, we found that exposure of cells sensitive to PFI-1 cause cell-cycle arrest. In contrast, the cell cycle in PFI-1–insensitive cell lines (K-562) was not affected as shown by flow cytometry (Supplementary Fig. S4). Because c-Myc drives expression of a number of genes that are essential for cell-cycle progression (e.g., E2Fs, CDC25A, CDK2, CDK4, and Rb), the observed G1–S arrest might be a consequence of c-Myc depletion in sensitive MV4:11 but not K-562 cells (28–31).

**PFI-1 downregulates Aurora B and attenuates H3S10 phosphorylation**

BRD4 is one of the few transcriptional regulators that are recruited to chromatin during mitosis providing a mechanism for transcriptional memory during cell division (14, 15, 22). Mitotic entry and progression are principally regulated by serine/threonine kinases of the Aurora family (16). Aurora kinases are highly expressed in diverse cancer types and are also frequently upregulated in leukemia (32, 33). In addition, Aurora B expression levels are modulated by BRD4 and are stimulated by c-Myc, whereas c-Myc stability is regulated by Aurora kinase–dependent degradation (21). We were therefore interested to study whether inhibition of BET BRDs by the inhibitors PFI-1 and (+)-JQ1 would affect Aurora B protein levels resulting in indirect inhibition of the Aurora B oncogene.

We found high expression levels in Western blots using Aurora B–specific antibodies in both MV4:11 and K-562 cells.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>GI50 [μmol/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV4:11</td>
<td>Childhood B-cell myelomonocytic acute leukemia (MLL/AF4)</td>
<td>1.5 ± 1</td>
</tr>
<tr>
<td>THP-1</td>
<td>Acute monocytic leukemia (MLL/AF9)</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>KASUMI-1</td>
<td>Acute myeloid leukemia with t(8;21) translocation (AML1-ETO)</td>
<td>0.8 ± 1</td>
</tr>
<tr>
<td>NALM-17</td>
<td>Pre-B blast ALL</td>
<td>8 ± 4</td>
</tr>
<tr>
<td>TOM-1</td>
<td>Ph1-positive ALL</td>
<td>3 ± 5</td>
</tr>
<tr>
<td>REH</td>
<td>Adult ALL</td>
<td>9 ± 4</td>
</tr>
<tr>
<td>SD-1</td>
<td>Epstein-Barr virus immortalizedimmortalized Ph-1 positive ALL</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>NALM-6</td>
<td>Pre-B ALL [t(5; 12) (q33; p13)]</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>NOMO-1</td>
<td>Acute monocytic leukemia (MLL/AF9)</td>
<td>3 ± 3</td>
</tr>
<tr>
<td>SEMK-H2</td>
<td>Acute monocytic leukemia (MLL/AF9)</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>RS-44-11</td>
<td>Acute monocytic leukemia (MLL/AF4)</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>KOCL-45</td>
<td>Acute monocytic leukemia (MLL/AF4)</td>
<td>10 ± 5</td>
</tr>
<tr>
<td>U937</td>
<td>Leukemic monocytic lymphoma</td>
<td>5 ± 3</td>
</tr>
<tr>
<td>PL21</td>
<td>Acute promyelolytic leukemia</td>
<td>10 ± 5</td>
</tr>
<tr>
<td>SUPB-15</td>
<td>Ph+ childhood B-cell ALL</td>
<td>11 ± 4</td>
</tr>
<tr>
<td>K-562</td>
<td>Erythroleukemia CML blast (Bcr-ABL)</td>
<td>&gt;20</td>
</tr>
</tbody>
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Aurora B often appeared as a double band possibly due to different phosphorylation states of the protein. Exposure of these cells to PFI-1 and (+)-JQ1 resulted in significant reduction of Aurora B protein levels after 8-hour exposure in the BET inhibitor–sensitive cell line MV4;11. In K-562 cells, a reduction of Aurora B has been observed after 4 hours but the protein levels were increased again after 8 hours. This is likely due to differences of BET inhibitors on Aurora B mRNA expression and Aurora B degradation in these 2 cell lines. As a consequence of Aurora B downregulation, we observed significantly reduced phosphorylation of the Aurora substrate histone 3 S10 (H3S10) in cells stained with H3S10p-specific antibodies (Fig. 6B); H3S10 phosphorylation was completely ablated in Western blots (Fig. 6C) in MV4;11 cells. Immunocytochemistry and Western blots showed that reduction of H3S10 phosphorylation levels were significantly less dramatic in K-562 cells than observed in MV4;11 cells. In conclusion, BRD4 inhibition results in significant Aurora B inactivation offering an alternative strategy for inhibition of this attractive kinase target.

Synthetic lethality of BET and Aurora kinase inhibition

As efficacious killing of tumor cells by Aurora inhibitors is often constrained by dose-limiting toxicity, we were interested if dual inhibition of BET proteins and Aurora kinases would be synergistic. We selected for these studies the potent and reversible small-molecule Aurora inhibitor VX-680 (MK-0457) that inhibits all 3 Aurora kinase isoforms with low nanomole potency (0.6, 18, and 4.6 nmol/L for Aurora A, Aurora B, and Aurora C, respectively). This inhibitor has shown in vivo efficacy in xenograft models in human AML and other cancers and has entered clinical testing (34–37). Aurora kinases interact with many key regulators of the cell cycle and cell survival, including p53, cyclin B, and Cdc2. As a consequence, inhibition of Aurora kinases affects different stages of the cell cycle: Aurora A inhibition has been associated with G2–M phase...
arrest, whereas Aurora B inhibition leads to failure in cell division, abnormal exit from mitosis, polyploidy cells, and ultimately induction of apoptosis. We observed at low inhibitor concentration (lower than 20 nmol/L) cell-cycle arrest in G2-M phase consisted with Aurora A inhibition. A higher concentration, VX680, caused predominantly G0–G1 arrest. Apoptosis was observed at concentrations higher than 160 nmol/L (Supplementary Fig. S5). Interestingly, combination PFI-1 or (−)-JQ1 and VX-680 at concentration that do not cause observable toxicity as single agents led to strong cancer cell toxicity (Fig. 7A). Intriguingly, the optimal concentration of VX-680 was 40 nmol/L for both BET inhibitors tested. At this concentration, the G2–M arrest observed at lower VX-680 is released leading to sensitization of the tested cell lines to dual Aurora and BET inhibition. At higher VX-680 concentration, however, this synergistic effect is lost. It is therefore likely that the strong induction of apoptosis at 40 nmol/L VX-680 with both BET inhibitors is highly dependent on differences in BET-dependent gene expression and cell-cycle effects induced by Aurora kinase inhibitors. No synergy between VX-680 and BET inhibitors was observed in K-562 cells that are insensitive to BET inhibition (data not shown).

BET inhibition causes downregulation of Aurora B in vivo

To study downregulation of Aurora B in vivo, we established a xenograft model of high-risk primary childhood B-cell ALL. Pharmacokinetics studies in mice suggested that the necessary effective dose of PFI-1 cannot be obtained in vivo (23) which prevented us from using PFI-1 for long-term in vivo studies in mice and (−)-JQ1 was therefore used. Engraftment was monitored by staining peripheral blood samples for human and mouse CD45. After 5 weeks of engraftment in NOG mice, the level of hCD45 was at least 1% and mice were treated for 3 weeks with 50 mg/kg (−)-JQ1 as described (10). Bone marrow was extracted from the femur of tumor-bearing mice and stained with specific antibodies for Aurora B and c-Myc. The immunofluorescent images revealed strong downregulation of both Aurora B as well as c-Myc (Fig. 7B) showing that BET...

Figure 5. Western blots showing induction of apoptosis and downregulation of c-Myc in MV4;11 but not K-562 cells. Shown are Western blot data on the BET inhibitor sensitive cell line MV4;11 and the insensitive cell line K-562 (cleavage of procaspase 7, PARB activation, BAD phosphorylation, and c-Myc downregulation) using cell extracts of PFI-1-treated cells after 0, 24, and 48 hours of incubation times.

Figure 6. Downregulation of Aurora B. A, Western blot analysis of Aurora B expression in MV4;11 and K-562 cells treated with PFI-1 and (−)-JQ1. B, immunohistochemistry showing reduction of phosphorylation of the Aurora B substrate H3S10. DNA is stained with 4’, 6-diamidino-2-phenylindole (DAPI). C, Western blot analysis of phosphor H3S10 after PFI-1 and (−)-JQ1 treatment of MV4;11 and K-562 cells.
inhibition significantly reduces Aurora B levels in this in vivo model.

**PFI-1 and JQ1 dissociate BRD4 from HOXA9 and promotes differentiation**

Evolutionarily conserved HOX genes are transcription factors that play an important role during hematopoiesis in regulating apoptosis, receptor signaling, differentiation, motility, and angiogenesis. Aberrant expression of HOX genes has been implicated in the development of leukemia and other cancers (38–40). During hematopoietic cell development, clusters of HOX genes are highly expressed in primitive hematopoietic cells and in poorly differentiated leukemic cells, whereas they are largely downregulated in differentiated cells. In particular, HOXA9 has been identified as a marker of poor prognosis in patients with acute myeloid leukemia (41), and overexpression of HOXA9 leads to immortalization of bone marrow cells and development of leukemia in mice (42). We were therefore interested to know whether BRD4 inhibition directly influences HOXA9 expression and induces differentiation of primary leukemic blasts. ChIP assays showed that both (+)-JQ1 as well as PFI-1 displace BRD4 from the HOXA9 promoter region (Fig. 7C, Supplementary Fig. S6). Quantitative PCR showed that HOXA9 mRNA is upregulated after 4 hours but strongly downregulated after incubation times of 8 and 24 hours in PFI-1–treated MV4;11 cells but not in BET inhibitor–insensitive K-562 cells that follow, however, the same biphasic pattern of the detected mRNA levels (Fig. 7D). The delayed response of HOXA9 transcriptional downregulation suggests an indirect mechanism that is possibly regulated by reduced c-Myc levels. As PFI-1 inhibits all BET family members, differences in BET protein levels or levels of the target genes may...
result in the different overall response of HOX9 expression in these 2 cell lines. In agreement with the known key function of HOX9 in suppressing differentiation, murine MLL-AF9 leukemic blasts treated with PFI-1 showed rapid differentiation into cells with polymorphonuclear neutrophil-like appearances (Fig. 7E).

Discussion

This study presents a chemically diverse BET inhibitor PFI-1 with high potency and selectivity for this subfamily of BRD-containing proteins. Here, we used PFI-1 to study the role of BET proteins in acute leukemia and in regulating HOX9 expression as well as Aurora B kinase activity. Inhibition of BET BRDs results in the change of transcription of diverse target genes, many of them linked to cellular proliferation and prevention of apoptosis. The set of BET-regulated target genes is highly correlated in sensitive cell lines. For instance, in the study by Dawson and colleagues (13), the top 100 genes that were found to be decreased in the sensitive MLL cell lines MOLM-13 and MV4;11 overlapped and contained many known MLL target genes such as key regulators of proliferation (MYC, CDK6) and antiapoptotic genes (BCL2), suggesting that BET proteins are required for efficient expression of genes driven by oncogenic MLL-fusion proteins. Expression of these genes was less affected in inhibitor insensitive K-562 cells expressing the BCR-ABL fusion gene product. A recent study by Mertz and colleagues has shown that MYC expression was affected by BET inhibition only in the context of natural, chromosomally translocated or amplified gene loci, but not if expression is driven by non-endogenous promoters or viral insertions providing a possibility for stratification of BET-sensitive cancer types (27).

In this study, we have also shown that BET inhibition results in significant downregulation of Aurora B kinase in vitro as well as in vivo. However, whether Aurora B expression is directly mediated by BET or indirectly through downregulation of MYC expression remains to be shown. A recent report showed that knockdown of the BET family member BRD4 results in down-regulation of Aurora B expression, whereas exogenous overexpression of BRD4 increases Aurora B protein levels (43). In synchronized cells, Aurora B protein levels have been significantly reduced during mitosis after RNAi knockdown of BRD4 (43) and abnormal chromosomal segregation has been observed in BRD4-depleted primary human foreskin keratinocytes leading to high frequency of binuclear tetraploid and octoploid nuclei (43). Aurora B activity is also tightly linked to c-Myc function. Yang and colleagues have recently shown that the pan-Aurora inhibitor VX-680 preferentially kills cells that overexpress c-Myc (44) and that both oncogenes are frequently amplified in colorectal carcinomas and medulloblastomas (45, 46). Here, we showed that VX-680 inhibition is strongly synergistic with BET inhibition showing effective induction of apoptosis at concentration that did not show any cytotoxic effects of the single agents. Interestingly, synergism of VX-680 has also been observed with the HDAC inhibitor vorinostat as a result of reactivated proapoptotic genes and enhanced cancer cell death. It is likely that suppression of antiapoptotic and reactivation of proapoptotic genes that have been reported for BET inhibitors (13) leads to the strong induction of apoptosis that have been observed in our synergy study.

The 8 BRDs present in the 4 human BET family members share high sequence similarity in their acetyl-lysine-binding sites making the design of target selective inhibitors a challenging task. Although we believe that it will be difficult to achieve high selectivity for any of the isoforms, we observed that all first and second BRDs contain a set of diverse residues. These differences could be explored for the development of subdomain-specific inhibitors, which is an ongoing research activity in our laboratory.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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