Selective Targeting of Bromodomains of the Bromodomain-PHD Fingers Family Impairs Osteoclast Differentiation

Julia C. Meier,† Cynthia Tallant,† Oleg Fedorov,† Hanna Witwicka,‡ Sung-Yong Hwang,‡ Ruud G. van Stiphout,∥ Jean-Philippe Lambert,⊥ Catherine Rogers,† Clarence Yapp,† Brian S. Gerstenberger,∥ Vita Fedele,† Pavel Savitsky,† David Heidenreich,+ Danette L. Daniels,▼ Dafydd R. Owen,### Paul V. Fish,○ Niall M. Igoe, ◕ Elliott D. Bayle,○ Bernard Haendler,♦ Udo C.T. Oppermann,¶ Francesca Buffa,∥ Paul E. Brennan,○ Susanne Müller,§ Anne Claude Gingras,⊥ Paul R. Odgren,‡ Mark J. Birnbaum,∥ and Stefan Knapp*,†,§,

†Target Discovery Institute and Structural Genomics Consortium, Oxford University, Oxford, United Kingdom
‡Department of Cell and Developmental Biology, University of Massachusetts Medical School, Worcester, Massachusetts, United States
§Buchmann Institute for Life Sciences (BMLS), Riedberg Campus, 60438 Frankfurt am Main, Germany
∥Department of Oncology, Oxford University, Old Road Campus Research Building, Oxford OX3 7DQ, United Kingdom
⊥Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario MSG 1X5, Canada
#Pfizer Worldwide Medicinal Chemistry, 610 Main Street, Cambridge, Massachusetts 02139, United States
∇Promega Corporation, Madison, Wisconsin, United States
○Department of Pharmaceutical & Biological Chemistry, UCL School of Pharmacy, University College London, 29-39 Brunswick Square, London, WC1N 1AX, United Kingdom
❖Drug Discovery, Bayer Pharma AG, Müllerstrasse 178, D-13353 Berlin, Germany
♣Botnar Research Centre, Oxford University, Oxford, United Kingdom
♦Goethe-University Frankfurt, Institute of Pharmaceutical Chemistry, Riedberg Campus, 60438 Frankfurt am Main, Germany
▲Department of Molecular Genetics, University of Toronto, Toronto, Ontario M5S 1A8, Canada
□Department of Biology, Merrimack College, North Andover, Massachusetts, United States
▲German Cancer Network (DKTK), Frankfurt site, 60438 Frankfurt am Main, Germany

Supporting Information

ABSTRACT: Histone acetyltransferases of the MYST family are recruited to chromatin by BRPF scaffolding proteins. We explored functional consequences and the therapeutic potential of inhibitors targeting acetyl-lysine dependent protein interaction domains (bromodomains) present in BRPF1-3 in bone maintenance. We report three potent and selective inhibitors: one (PFI-4) with high selectivity for the BRPF1B isoform and two pan-BRPF bromodomain inhibitors (OF-1, NI-57). The developed inhibitors displaced BRPF bromodomains from chromatin and did not inhibit cell growth and proliferation. Intriguingly, the inhibitors...
Acetylation of histones and other nuclear proteins is a key mechanism regulating gene expression, and aberrant acetylation has been linked to a wide range of diseases. Histone acetylation is introduced by histone acetyltransferases (HATs) that transfer an acetyl moiety to the ε-amino group of lysine residues. HATs have usually broad substrate specificity in vitro. In contrast, in vivo, HAT substrate specificity is dramatically enhanced by scaffolding proteins that activate HATs and target them to specific chromatin sites.

Bromodomains are evolutionarily highly conserved protein–protein interaction modules that act as acetyl-lysine dependent epigenetic reader domains. The human proteome encodes 61 diverse bromodomains that are present in 46 proteins including the HATs CREBBP/EP300 and PCAF/GCN5. Bromodomains share a conserved fold that comprises a left-handed bundle of four α-helices (αZ, αA, αB, αC), linked by loop regions of variable length (ZA and BC loops), which line the KAc binding site and determine binding specificity. Due to its excellent druggability, the bromodomain acetyl-lysine binding pocket has emerged as an attractive site for the development of protein interaction inhibitors. Highly potent and selective inhibitors have been developed for BET (BRD2, BRD3, BRD4, BRDT) bromodomains, and a number of BET inhibitors have now entered clinical testing. While to date most efforts have focused on BET inhibitor development, recent publications have demonstrated that non-BET bromodomains can also be selectively targeted.

A first inhibitor specific for the BRPF1 bromodomain has been recently disclosed, and inhibitors that showed dual activity for residues. HATs have usually broad substrate specificity in vitro. However, phenotypic consequences of inhibiting protein interactions mediated by BRPF bromodomains have not been reported so far.

The MYST (MOZ, TIF2/Sas3, SAS2 and Tip60) family of lysine acetyl-transferases form signaling complexes with heterotetrameric core structures comprising a MYST family member, the ING tumor suppressor, hEAF6 (an EPC enhancer of polycomb)-associated protein, and a central scaffolding protein of the BRPF (Bromodomain-PHD fingers) family. In higher eukaryotes, the BRPF family contains three members (BRPF1, BRPF2 (also called BRD1), and BRPF3) with conserved domain architecture of two N-terminal PHD domains linked by a Zn2+ knuckle (PZP [PHD−Zn knuckle−PHD] domain), a bromodomain, and a C-terminal PWWP domain. The BRPF PHD domains target unmethylated histone H3. The PWWP domains recognize the H3K36me3 mark, and the bromodomains preferentially interact with H2AK5ac, H4K12ac, and H3K14ac. BRPF1 associates with MOZ/MORF, assembling a signaling complex that plays a role in maintaining anterior HOX gene expression during development. MOZ is frequently translocated in acute myeloid leukemia (AML), and it is required for hematopoietic stem cell maintenance. Biochemical studies have shown that BRPF1 still interacts with and enhances the transcriptional potential of the leukemic MOZ-TIF2 fusion protein. BRPF2 preferentially associates with HBO1, assembling a chromatin complex required for global acetylation of H3K14ac. HBO1/BRPF2 plays a key role in the regulation of erythropoiesis. In mice, deletion of the BRPF2 gene results in severe anemia due to impaired fetal liver erythropoiesis. In addition, polymorphism in BRPF2 has been recently linked to bipolar disorder and schizophrenia. The related JADE scaffolding proteins that lack the C-terminal bromo and PWWP domains can replace BRPF2 in the HBO1 complex. Intriguingly, this exchange in scaffolding protein determines which histone tail is acetylated: the JADE complex directs HBO1 toward the H4 tail, whereas BRPF2 confers high selectivity for H3. The central role of reader domains in MYST complexes and the key role of these complexes in hematopoiesis prompted us to study the consequences of pharmacological targeting of BRPF readers of the bromodomain family in monocyte differentiation, with osteoclasts representing a particularly well-studied and clinically relevant monocyte-derived lineage. Here, we report that selective pharmacological inhibition of BRPF bromodomains but not of the BRPF1B bromodomain alone strongly impaired RANKL-induced differentiation of murine and human primary monocytes into bone resorbing osteoclasts. Genomewide mRNA expression analysis showed that panBRPF bromodomain inhibition suppressed transcriptional programs required for osteoclastogenesis, establishing a role of BRPF family members in bone degradation. The data indicate that interactions mediated by BRPF bromodomains play a central role in bone maintenance and may be attractive targets for the development of drugs preventing osteoporosis and metastasis or cancer induced osteolysis.

RESULTS AND DISCUSSION

The human BRPF family (BRPF1, BRPF2, and BRPF3) shares a conserved domain architecture and a high degree of sequence homology within their bromodomains. Interestingly, we found that alternative splicing generates two BRPF1 isoforms (A and B). The longer BRPF1A harbors a six-residue insert in the ZA-loop that prevented binding to histone peptides as well as inhibitors. However, phenotypic consequences of inhibiting protein interactions mediated by BRPF bromodomains have not been reported so far.

The MYST (MOZ, TIF2/Sas3, SAS2 and Tip60) family of lysine acetyl-transferases form signaling complexes with heterotetrameric core structures comprising a MYST family member, the ING tumor suppressor, hEAF6 (an EPC enhancer of polycomb)-associated protein, and a central scaffolding protein of the BRPF (Bromodomain-PHD fingers) family. In higher eukaryotes, the BRPF family contains three members (BRPF1, BRPF2 (also called BRD1), and BRPF3) with conserved domain architecture of two N-terminal PHD domains linked by a Zn2+ knuckle (PZP [PHD−Zn knuckle−PHD] domain), a bromodomain, and a C-terminal PWWP domain. The BRPF PHD domains target unmethylated histone H3. The PWWP domains recognize the H3K36me3 mark, and the bromodomains preferentially interact with H2AK5ac, H4K12ac, and H3K14ac. BRPF1 associates with MOZ/MORF, assembling a signaling complex that plays a role in maintaining anterior HOX gene expression during development. MOZ is frequently translocated in acute myeloid leukemia (AML), and it is required for hematopoietic stem cell maintenance. Biochemical studies have shown that BRPF1 still interacts with and enhances the transcriptional potential of the leukemic MOZ-TIF2 fusion protein. BRPF2 preferentially associates with HBO1, assembling a chromatin complex required for global acetylation of H3K14ac. HBO1/BRPF2 plays a key role in the regulation of erythropoiesis. In mice, deletion of the BRPF2 gene results in severe anemia due to impaired fetal liver erythropoiesis. In addition, polymorphism in BRPF2 has been recently linked to bipolar disorder and schizophrenia. The related JADE scaffolding proteins that lack the C-terminal bromo and PWWP domains can replace BRPF2 in the HBO1 complex. Intriguingly, this exchange in scaffolding protein determines which histone tail is acetylated: the JADE complex directs HBO1 toward the H4 tail, whereas BRPF2 confers high selectivity for H3. The central role of reader domains in MYST complexes and the key role of these complexes in hematopoiesis prompted us to study the consequences of pharmacological targeting of BRPF readers of the bromodomain family in monocyte differentiation, with osteoclasts representing a particularly well-studied and clinically relevant monocyte-derived lineage. Here, we report that selective pharmacological inhibition of BRPF bromodomains but not of the BRPF1B bromodomain alone strongly impaired RANKL-induced differentiation of murine and human primary monocytes into bone resorbing osteoclasts. Genomewide mRNA expression analysis showed that panBRPF bromodomain inhibition suppressed transcriptional programs required for osteoclastogenesis, establishing a role of BRPF family members in bone degradation. The data indicate that interactions mediated by BRPF bromodomains play a central role in bone maintenance and may be attractive targets for the development of drugs preventing osteoporosis and metastasis or cancer induced osteolysis.

To enable identification of BRPF bromodomain inhibitors, we developed an ALPHAScreen (amplified luminescent proximity homogeneous assay) assay using a tetra-acetylated histone 4 peptide (H4K5acK8acK12acK16ac) and his6-tagged recombinant BRPF bromodomains. Screening of an in-house bromodomain targeted library and fragment sets resulted in the identification of a number of inhibitors that carried either the benzoimidazolone or the dimethylquinolinone core structure. Purchasing and optimization by synthetic medicinal chemistry efforts led to the development of the potent benzoimidazolone-based inhibitors 01, PFI-4, and the dimethylquinolinone NI-57 (Figure 2A). A detailed discussion of the SAR (structure–activity relationship) will be reported elsewhere. Using dose response ALPHAScreen assays, the developed chemical probes showed potencies for BRPF1B (IC50) of 270 nM, 172 nM, and 114 nM for 01, PFI-4, and NI-57, respectively. These data correlated well with temperature shift and isothermal titration calorimetry data (ITC), but ALPHAScreen underestimated somewhat the affinity of...
PFI-4 for the BRPF1B isoform, which had a $K_D$ of 13 ± 1 nM using ITC (Supporting Information Tables 1–3).

Next, we used temperature shift assays ($\Delta T_m$) to evaluate the family wide selectivity of the three developed chemical probes using a comprehensive panel of 49 diverse bromodomains. OF-1 showed significant $\Delta T_m$ shifts within the BRPF family identifying this inhibitor as a pan-BRPF inhibitor, but also weak interactions (2.1°C) were observed for BRD4(1). $\Delta T_m$ shifts of 1°C were observed for other bromodomains such as other BET family members, TRIM24 (TIF1α), and BRD9 (Figure 2B). However, such small $\Delta T_m$ shifts often represent false positive hits or very weak interactions. Indeed, ALPHAscreen did not reveal strong interactions of OF-1 with BRD4 and TRIM24 (TIF1α), with IC$_{50}$ values larger than 10 μM (Supporting Information Table 2). ITC determined a $K_D$ of 3.9 ± 0.3 μM for the first bromodomain of BRD4(1), thus 39-fold selectivity when compared to the BRPF1B isoform (Figure 2C). Using ITC, we determined $K_D$ values of 0.5 ± 0.06 μM and 2.4 ± 0.2 μM for BRPF2 and BRPF3, respectively. We did not identify any significant interaction outside subfamily IV for NI-57, which showed only weak $\Delta T_m$ shifts (~1°C) for the bromodomain present in BRD9, CREBBP, and EP300, suggesting excellent selectivity for the BRPF family. ITC revealed a $K_D$ of 0.031 ± 0.002 μM for BRPF1B (Supporting Information Table 3), in agreement with ALPHAscreen data (IC$_{50}$: 0.114 ± 0.061 μM). Finally, PFI-4 was highly selective for the BRPF1B isoform. $\Delta T_m$ screening against the bromodomain family detected only a weak temperature shift for the CECR2 bromodomain. ITC confirmed isoform selectivity for BRPF1B ($K_D$: 0.013 ± 0.001 μM), whereas BRPF2 interacted with this chemical probe with a $K_D$ of 0.775 ± 0.09 μM (60-fold selectivity) and CECR2, the only detected off-target outside family IV with only 2.35 ± 0.52 μM affinity, thus showing 180-fold selectivity.
BRD7 was detected in $T_m$ assays, but this interaction was not confirmed in alternative assays (DiscoverX bromoscan) probably due to the low intrinsic stability of the protein leading often to misleading DSF results (Figure 2D). It is interesting to note that in agreement with our peptide binding data, none of the inhibitors interacted with the BRPF1A isoform in temperature shift assays, consistent with this splicing isoform acting as a bromodomain inactivating variant with an inaccessible acetyl-lysine binding site. Screening of the developed probes against a diverse panel of potential off-targets (kinases, GPCRs) revealed no significant off-targets outside the bromodomain family (Supporting Information Table 4). In conclusion, medicinal chemistry and in vitro screening efforts led to the development of three potent chemical tools with good selectivity for the BRPF family as well as one highly isoform-selective chemical probe. Thus, this set of three chemical probes allows independent evaluation of phenotypic consequences of BRPF bromodomain inhibition as well as BRPF1B specific activities in cellular systems.

Following the analysis of inhibitor potency and selectivity in vitro, we set out to demonstrate cellular “on-target” activity of the three probe molecules. Since the developed inhibitors are anticipated to block chromatin association of BRPF, it is expected that the inhibitors would weaken the interactions of BRPF with histones and strongly inhibit the recruitment of isolated BRPF bromodomains to histones. To assess this, we developed a BRPF1A/B-Histone H3.3-nanoBRET (nano bioluminescence resonance energy transfer) assay, which measures the energy transfer from one NanoLuciferase coupled protein (BRPF1, donor) to the interacting HaloTag-protein labeled with a NanoBRET 618 fluorophore (histone H3.3 acceptor). Indeed, we observed dose-dependent displacement of BRPF1B but not of the BRPF1A isoform from histone H3.3 (Figure 3, Supporting Information Figure 1). Estimated IC$_{50}$ values were 0.07 ± 0.0034 and 0.24 ± 0.039 μM for NI-57 and PFI-4, respectively. We verified these data using FRAP (fluorescence recovery after photobleaching) assays. As expected from our selectivity screening data, PFI-4 led only to the dissociation of the bromodomain of BRPF1b, but not any of the full-length family members from histone H3.3 (Supporting Information Figure 1B). NI-57 displaced a GFP fusion construct where the bromodomain was triplicated as well as full-length GFP-BRPF2, but not inactivating bromodomain mutants from chromatin, which was indicated by significant reduction of recovery times in the presence of the inhibitor. Acetylation dependence of the interaction was demonstrated by adding the pan-HDAC inhibitor suberoylanilide hydroxamic acid (SAHA), which leads to a global increase in histone acetylation and therefore to stronger association of the BRPF bromodomains with histones (Figure 3B−D). Similar results were observed for OF-1 and PFI-4 (Supporting Information Figure 1). Thus, the nanoBRET and FRAP
experiments demonstrated that the developed inhibitors strongly inhibit BRPF1 and BRPF2 but not BRPF1A bromodomains in the nucleus.

Structural models of monoacetylated histone peptides H2AK5ac and H4K12ac have been published recently, revealing a canonical bromodomain acetyl-lysine interaction. However, we wanted to confirm the binding mode of peptides that we used in screening assays and for which we detected the tightest association with BRPF1B. In particular, we were interested in the consequences of the presence of multiple acetylation sites on histone recognition as well as the recognition of the histone H3 mark H3K14ac. We therefore cocrystallized BRPF1B with peptides harboring the H3K14ac and H4K5acK8ac mark. The H3K14ac complex revealed the canonical interaction of the acetyl-lysine with the BRPF1B bromodomain comprising the conserved hydrogen bond with N708 as well as the water-mediated hydrogen bond with Y665 and additional hydrogen bonds formed by the H3R17 side chain and the backbone carbonyl of the G650 (Supporting Information Figure 2A–C).

It is interesting to note that in the H3K14ac complex the peptide reversed its orientation when compared to complexes of the same mark with the bromodomain of BA2Z2B. Co-crystallization of the diacetylated peptide H4K5acK8ac revealed that in contrast to cocrystal structures with BRD4 only H4K5ac interacted with the acetyl-lysine binding site, probably due to steric constraints of the bulky residue F714 preventing simultaneous interaction of two acetylated lysines in BRPF1B (Figure 4A).

In the cocrystal structure, the H4K8ac side-chain was oriented toward the surface but in close proximity to an area of strongly positive electrostatic potential. It is therefore likely that neutralization of the positive charge of the lysine by acetylation contributes favorably to the interaction with this bromodomain.

We cocrystallized OF-1 as well as PFI-4 to confirm the acetyl-lysine mimetic binding mode suggested by our peptide displacement screening assays and to elucidate the structural mechanisms of the observed selectivity. As expected, the benzimidazolone acted as an acetyl-lysine mimetic moiety forming in the BRPF1B complex the canonical hydrogen bond between the conserved asparagine (N708) and the characteristic water-mediated hydrogen bond with Y665 (Figure 4B,D). The inhibitor was further stabilized by a number of hydrophobic interactions with lipophilic groups located at the rim of the Kac binding site. The sulphonamide linker caused a 90° bend, positioning the bromo-methylphenyl ring on top of F714, allowing an aromatic edge-face stacking interaction and hydrophobic contacts with I713. Comparison with the BRPF2-OF1 complex showed conservation of the binding mode, but the bromo-methylphenyl ring assumed a position that is turned away from F714 due to rotation of the phenyl ring (Supporting Information Figure 4D).

Sequence conservation in the acetyl-lysine binding pocket of BRPF1B and BRPF2 is high, but the BC-loop residue I713 and the central ZA-loop residue P658 positions are substituted by...
V647 and S592 in BRPF2. This creates a larger and shallower binding pocket, resulting in repositioning of OF-1. ITC data suggested that higher affinities of the developed inhibitors for the BRPF1B isoform are mainly due to a more favorable binding enthalpy (Supporting Information Table 3). It is therefore likely that the less efficient stacking of the bromo-methylphenyl ring with F714 as well as the lack of interaction of the halogen atom with the adjacent binding pocket formed by I713 are the main reasons for the 5-fold weaker potency of OF-1 for BRPF2. Comparison with the BRPF1B-PFI-4 complex revealed that the amide linkage with the benzoimidazolones ring system does not allow orientation of the methoxybenzamide toward F714 (Figure 4D, Supporting Information Figure 2E,F). The orientation of the PFI-4 methoxybenzamide ring is additionally stabilized by the presence of an intramolecular hydrogen bond, resulting in an orientation toward the ZA loop. Together with the 6-pyrrolidine substituent, the inhibitor shows remarkable shape complementarity with the BRPF1B acetyl-lysine binding site, explaining the high potency for this target. Crystallographic data and refinement statistics are summarized in Supporting Information Table 5.

To understand the complexity of the BRPF signaling network, we used proximity-dependent protein biotinylation (BioID) to identify physiologically relevant BRPF protein interactions in living cells using HEK293 as a model.36 The experiments revealed the canonical BRPF core complex structure, comprising MYST acetyltransferases as well as MYST/Esa1-associated factor 6, which was found preferentially associated with BRPF3, and the general complex components ING4/5, in addition to other associated proteins that were often isoform specific (Figure 5A). ING4/5 associated with all BRPF family members, but it is interesting to note that MYST family members preferentially associate with specific BRPF isoforms. HBO1 (KAT7) was reported to preferentially interact with BRPF2, but significant association with BRPF3 was also detected. KAT6A (MOZ) preferentially associated with BRPF1B, in agreement with published data,26 but MORF (KAT6B) also showed significant interaction with a short isoform of BRPF2. Also interesting was the presence of Ser/Thr phosphatases (PPP1CC) and their regulators as well as the members of the casein kinase family, which were found in BRPF1B complexes, suggesting crosstalk with phosphorylation dependent signaling events (Supporting Information Figure 3). Epigenetic mechanisms play an important role in patterning and differentiation processes, and BRPF and its associated HATs have been particularly associated with differentiation of hematopoietic cells. Given our interest in bone biology, and the hematopoietic origin of the osteoclast, we investigated the potential role of the targeted bromodomains in osteoclastogenesis in mouse and human cells. Bone marrow mononuclear cells were isolated from wild-type mice, cultivated for 5 days in the presence of CSF-1, and replated in the presence of RANKL for differentiation into osteoclasts,28 either with DMSO as a control or with varying doses of the BRPF inhibitors. OF-1 and NI-57 both caused significant reductions in the number of multinucleated tartrate-resistant acid phosphatase (TRAP) positive cells (Figure 5B, Supporting Information Figure 4). To understand the molecular basis for this observation, we investigated the expression of several osteoclast marker genes using qPCR. As expected, we found significant reductions of...
TRAP, the late-phase osteoclast marker cathepsin K (CATK), the proton generator carbonic anhydrase II (CA2), the key transcription factor NFATC1, and OC-STAMP, an essential gene involved in cell–cell fusion (Figure 5C). Suppression of gene expression by OF-1 was particularly strong at day 2 after RANKL-induced differentiation. Interestingly, the dominant negative form BRPF1A exhibited significantly decreased RNA expression levels, whereas the active acetyl-lysine binding isoform BRPF1B was not affected (Supporting Information Figure 4B). Additionally, BRPF bromodomain inhibitors had no effect on the proliferation of murine monocytic RAW246.7 cells and primary bone marrow cells, suggesting that the developed inhibitors are not cytotoxic (Supporting Information Figure 4). At the protein level, we also observed strong down-regulation of NFATC1 (Figure 5D). To further investigate other osteoclast-specific effects of the BRPF inhibitors on gene transcription, we profiled genome-wide expression with Illumina MouseWG-6 v2.0 Expression BeadChips at different time points. Significant differences (α = 0.05) in gene expression were observed at 48 and 72 h but not at 24 h of treatment (Figure 6). Pathway analysis (reactome.org) of the top 25 genes showed that differentially expressed genes were linked either to osteoclast differentiation or...
to lipid metabolism, thus two pathways relevant to osteoclast differentiation and function. For example, RANKL-induced expression of Ppap2a and SphK1 which play central roles in sphingolipid de novo biosynthesis, were significantly down-regulated by OF-1. These data suggest that the fusion process from macrophages to bone-resorbing “osteoclast-like” cells is interrupted by bromodomain inhibition as well.

To ensure that the inhibitors also have activity on human cells, we used human peripheral blood from healthy donors. Consistent with the results in murine cells, we observed dose-dependent suppression of osteoclast differentiation in the presence of all three inhibitors and RANKL (Figure 7A). OF-1 was the only inhibitor to completely suppress the fusion into multinucleated “osteoclast-like” cells. This suggests that during osteoclastogenesis, other BRPF family members may, at least in part, functionally replace BRPF1B, which was the only BRPF family member inhibited by PFI-4 at the concentrations tested (Figure 7B). To study this process in more detail, we performed fluorescence microscopy analyses for beta-actin, VNR (vitrinnectin receptor; $\alpha_\beta_3$ integrin), and DNA (DAPI), to visualize the cytoskeleton with the osteoclast specific actin rings that assemble podosomes as well as nuclei. The actin ring is a hallmark of osteoclasts which forms a seal with the bone surface to create a protected compartment for bone resorption by HCl and proteases. Treatment with all three inhibitors led to striking decreases of F-actin rings. We also analyzed bone-resorbing activity, first by seeding the cells onto a bone mineral substrate (hydroxyapatite, HA; Osteosurface assay, Corning). Both OF-1 and NI-57 led to a significant reduction in pit formation (Figure 7C), consistent with observed decreases in differentiation. Second, cells were seeded on dentine slices, a more biologically complete substrate. As with the HA plates, resorption of dentine was also markedly reduced, including a complete block of resorption by 1 $\mu$M OF-1 (Figure 7D). Consistent with this loss of activity, osteoclast marker genes CA2, CATK, NFATC1, and ACPS (TRAP) were also down-regulated using all three BRPF inhibitors (Figure 7E and F). Matrix metalloproteinase 9 (MMP9, type IV collagenase) is a key protease secreted by osteoclasts for matrix degradation. To determine whether BRPF bromodomain inhibition leads to down regulation of MMP9, we used an ELISA to determine the protein levels in the supernatant of primary osteoclasts before and after exposure to PFI-4, OF-1, and NI-57. We found that all three inhibitors led to significant reductions in MMP9 secretion (Figure 7G). Apart from its role in normal osteoclast function, the suppression of MMP9 secretion by the studied BRPF bromodomain inhibitors may also have important implications for targeting cancer metastasis in filtration into bone, since MMP9 secretion is markedly upregulated during progression toward invasive tumors due to its central role in stromal remodelling.37

The developed three chemical probes for the bromodomain of BRPF led to the identification of the role of this protein interaction domain in regulating osteoclastogenesis, suggesting a key role of protein acetylation in regulating this process. Previously, Lamoureux et al. demonstrated that panBET inhibition also attenuates osteoclast differentiation.38 However, in contrast to BET inhibitors, the inhibition of BRPF bromodomains did not result in antiproliferative effects or cytotoxicity. Inhibition of BRPF bromodomains may therefore be applicable for the prevention of bone loss, and the developed chemical probes will provide an excellent chemical starting point for translational studies. BRPF is widely expressed in a variety of tissue types, and the developed probes will help to elucidate further functions of these interesting epigenetic modulators.
METHODS

Protein Expression. All recombinant bromodomains were expressed as described in Filippakopoulos et al.35

BioLayer Interferometry (BLI). BLI experiments to determine binding kinetics were done using the Octet RED384 system by forteBIO using bromodomains of two splice isoforms of BRPF1A and BRPF1B that were biotinylated during recombinant expression using a BirA (Biotin transferase) overexpressing bacterial host (BL21 DE3) in conjunction with a C-terminal AVI-tag. Biotinylated proteins were immobilized on super streptavidin biosensors, which were subsequently quenched with L-biotin. Data analysis software provided by forteBIO was used to calculate binding constants from the interference data.

AlphaScreen. Assays were performed as described previously with minor modifications.39 Plates filled with 5 μL of the assay buffer followed by 7 μL of biotinylated peptide [H-YSGRGKacGGKacGGLGKacGGAKacRHRK(Biotin)−OH for BRD1, BRD4, BRPF1B, and BRPF3 or YQTARKSTGGK(ac)APRKQLATKAK(biotin)−OH for TIF1α] and His-tagged protein to achieve final assay concentrations of 25–100 nM depending on the dose–response curve for each individual protein.

Mice and Human Osteoclast Differentiation. Primary murine bone marrow mononuclear cells (BMMC) were obtained, differentiated, and stained for TRAP as previously described.40 Briefly, marrow cell suspensions from PBS flushed bones (two tibiae, two femora) from 2 to 3 week old mice were centrifuged at 1000 g for 5 min and plated in αMEM/10% FBS with low MCSF (10 ng/mL, human recombinant MCSF; Chiron) and incubated at 37 °C and 5% CO2. After 3 days, fresh medium containing high MCSF (75 ng/mL) was added for 2 more days, and then the medium was supplemented with RANKL (R&D Systems, Minneapolis) at 1 ng/mL unless otherwise noted, for the times indicated. Differentiation into osteoclasts was scored by counting multinucleated (three or more nuclei) TRAP-positive cells. Primary human peripheral blood (Oxford NHS Blood bank, NC10622) mononuclear cells (PBMCs) were collected from a Histopaque generated buffy coat after gradient
centrifugation at 20 min and 500g, brakes off. The CD14+ monocyte fraction was obtained by on-column CD14+MACS bead isolation (Miltenyi Biotech, Surrey, UK), washed twice with MACS buffer, and seeded at a density of 50,000/mL in rMEM/10%FCS supplemented with 25 ng/mL MCSF (Peprotech). After 6 days at 37°C, 5% CO2, treatment with either OF-1, NI-57, or PFI-4 with and without 50 ng/mL RANKL (Peprotech) was started. Media were changed and fresh compounds every 3–4 days. After 14–21 days, cells were fixed and stained for TRAP or lysed in RLT/2-mercaptoethanol RNA lysis buffer for RNA isolation.

**Immunofluorescence.** Fixed osteoclast-like cells were permabilized in 0.5% Triton X-100 at RT (RT) for 20 min. Blocking of non-specific binding was carried out with 3% FBS/PBS for at least 1 h at RT. Primary antibodies for VNR and F-actin were applied for 1 h in the dark, at RT. DAPI/PBS was added for 5 min, and fluorescence was measured by confocal microscopy (Zeiss).

**Bone Resorption Assays.** PBMCs were isolated and seeded onto either osteosurface assay plates (BD biosciences) or self-cut dentine slices from ivory (provided by Edward Hookway, Botnar institute). After 14 days of differentiation, cells were removed from osteosurface or dentine slices. The amount of osteoclast-mediated pits in the osteosurface was assessed by phase contrast. Dentine pits were imaged with confocal microscopy.

**Western Blot and ELISA.** Murine osteoclast-like cells were PBS washed, homogenized and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% NP-40. 0.5% Na-deoxycholate; 0.1% SDS; 2 mM EDTA; 10 mM NaF) containing protease inhibitors (Pierce, according to manufacturer’s specifications) with a 1 mL syringe and a 23-gauge needle. After centrifugation at 15 000 rpm at 4°C for 10 min, supernatants were frozen at −80°C. Protein concentrations were assayed using a Pierce BCA Kit, and 30 μg of protein per lane was subjected on 10% SDS gel. Proteins were electroblotted onto PVDF, and blots were probed overnight at 4°C (anti-NFATc1 (1:500); 7A6: sc-7294 mouse monoclonal, Santa Cruz) or anti-Lamin B1 (1:10 000; ab133741 rabbit monoclonal, Abcam). Secondary antibodies were HRP-conjugated sheep antimouse (1:5000 for Nfatc1; NA931V; GE Healthcare, Piscataway, NJ) or HRP-conjugated goat anti-rabbit (1:5000 for Lamin B1; # P0448; Dako, Carpenteria, CA). MMP9 secretion in the human osteoclast supernatant was determined by a MMP9 ELISA (R&D, DMP900) according to the manufacturer’s instructions. The supernatant (50 μL/well) was collected, frozen at −20°C, and 100-fold diluted for the assay. Absorbance was measured at 440 nm.

**Microarray and Bioinformatics Analysis.** Murine bone marrow stromal cells were differentiated into osteoclast-like cells as described above. Cells were PBS-washed and lysed in RLT-Buffer (including 2-Mercaptoethanol, Qiagen, UK), and RNA was isolated via RNeasy Plus Mini Kit (Qiagen, UK). After confirmation of RNA quality (RIN = 2 ± 0.1), cDNA synthesis of approximately 1000 ng of RNA with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, UK) was carried out. A total of 500 ng/µL cDNA was subjected to MouseWG-6 v2.0 Expression BeadChips (Illumina) at the Department of Pathology, University of Cambridge. Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-4155. Gene expression data underwent log2 transformation and quantile normalization. Genes were filtered based on their variation across the experiments, i.e., excluding genes with a standard deviation of expression lower than 0.1. For genes with multiple probes, only the highest variable probe was selected for further analysis. Differently expressed genes were selected using LIMMA (Linear Models for Microarray Data) in a paired analysis for the replicates. This method resulted in p-values adjusted for multiple testing using the Benjamini–Hochberg procedure, and α = 0.01 was used to define significance. Microarray analyses were performed in R (v3.0.1) using the packages Limma (3.16.8) for differential expression and Venn diagram (1.6.7), plotrix (3.5–7), gplots (3.4.1), and ggplot (2.14.1) for visualization. Pathway analysis of the top 25 differentially expressed genes with a Jaccard distance coefficient of p > 0.05 (Reactome.org) was performed. The REACT_111217 pathway (metabolism of lipids and lipoproteins) with at least more than four enriched genes was set as the most significant (entities p-value 0.045). Additional methods’ descriptions are available in the Supporting Information.

**REFERENCES**

(1) Falkenberg, K. J., and Johnstone, R. W. (2014) Histone deacetylases and their inhibitors in cancer, neurological diseases and immune disorders. Nat. Rev. Drug Discovery 13, 673–691.

(2) Verdin, E., and Ott, M. (2014) 50 years of protein acetylation: from gene regulation to epigenetics, metabolism and beyond. Nat. Rev. Mol. Cell Biol. 16, 258–264.

(3) Filippakopoulos, P., and Knapp, S. (2012) The bromodomain interaction module. FEBS Lett. 586, 2692–2704.
(4) Vidler, L. R., Brown, N., Knapp, S., and Hoelder, S. (2012) Druggability analysis and structural classification of bromodomain acetyl-lysine binding sites. J. Med. Chem. 55, 7346–7359.

(5) Filippakopoulos, P., Qi, J., Picaud, S., Shen, Y., Smith, W. B., Fedorov, O., Morse, E. M., Keates, T., Hickman, T. T., Felletar, L., Philpott, M., Munro, S., McKeown, M. R., Wang, Y., Christie, A. L., West, N., Cameron, M. J., Schwartz, B., Heightman, T. D., La Thangue, N., French, C. A., Wiest, O., Kung, A. L., Knapp, S., and Bradner, J. E. (2010) Selective inhibition of BET bromodomains. Nature 468, 1067–1073.

(6) Picaud, S., Da Costa, D., Thanasopoulos, A., Filippakopoulos, P., Fish, P. V., Philpott, M., Fedorov, O., Brennan, P., Bunnage, M. E., Owen, D. R., Bradner, J. E., Taniere, P., O’Sullivan, B., Muller, S., Schwaller, J., Stankovic, T., and Knapp, S. (2013) PFI-1, a highly selective protein interaction inhibitor, targeting BET Bromodomains. Cancer Res. 73, 3336–3346.

(7) Nicodem, E., Jeffrey, K. L., Schaefer, U., Beinke, S., Dewell, S., Chung, C. W., Chandwani, R., Marazzi, I., Wilson, P., Coste, H., White, J., Kirilovsky, J., Rice, C. M., Lora, J. M., Prinjha, R. K., Lee, K., and Tarakhovsky, A. (2010) Suppression of inflammation by a synthetic histone mimic. Nature 468, 1119–1123.

(8) Dawson, M. A., Prinjha, R. K., Dittmann, A., Giotopoulos, G., Bantscheff, M., Chan, W. I., Robson, S. C., Chung, C. W., Hofmann, A. F., Kessler, D., Knapp, S., Schuler, R. J., and Watson, R. J. (2014) LP99: Discovery and Synthesis of the First Selective BRD7/9 Bromodomain Inhibitor. J. Med. Chem. 59, 4642–4747.

(9) Filippakopoulos, P., and Knapp, S. (2014) Targeting bromodomains: epigenetic readers of lysine acetylation. Nat. Rev. Drug Discovery 13, 337–353.

(10) Clark, P. G., Vieira, L. C., Tallant, C., Fedorov, O., Singleton, D. C., Rogers, C. M., Monteiro, O. P., Bennett, J. M., Barioni, R., Muller, S., Daniels, D. L., Mendez, J., Knapp, S., Brennan, P. E., and Dixon, D. J. (2015) LP99: Discovery and Synthesis of the First Selective BRD7/9 Bromodomain Inhibitor. Angew. Chem. Int. Ed. 54, 6217–6221.

(11) Chen, P., Chaikuad, A., Bamborough, P., Bantscheff, M., Bountra, C., Chung, C. W., Fedorov, O., Grandi, P., Jung, D., Lesniak, R., Lindon, M., Muller, S., Philpott, M., Prinjha, R., Rogers, C., Selenski, C., Tallant, C., Werner, T., Willson, T. M., Knapp, S., and Dreyer, D. H. (2016) Discovery and Characterization of GSK2801, a Selective Chemical Probe for the Bromodomains BA2ZA and BA2ZB. J. Med. Chem. 59, 1410.

(12) Drouin, L., McGrath, S., Vidler, L. R., Chaikuad, A., Monteiro, O., Tallant, C., Philpott, M., Rogers, C., Fedorov, O., Liu, M., Akhtar, W., Hayes, A., Raynaud, F., Muller, S., Knapp, S., and Hoelder, S. (2015) Structure enabled design of BA2ZA-ICR, a chemical probe targeting the bromodomains of BA2ZA and BA2ZB. J. Med. Chem. 58, 2535–2559.

(13) Hay, D. A., Fedorov, O., Martin, S., Singleton, D. C., Tallant, C., Wells, C., Picaud, S., Philpott, M., Monteiro, O. P., Rogers, C. M., Conway, S. J., Rooney, T. P., Tumber, A., Yapp, C., Filippakopoulos, P., Bunnage, M. E., Muller, S., Knapp, S., Schuhfeld, C. J., and Brennan, P. E. (2014) Discovery and optimization of small-molecule ligands for the CBP/p300 bromodomains. J. Am. Chem. Soc. 136, 9308–9319.

(14) Theodoulou, N. H., Bamborough, P., Bannister, A. J., Becher, L., Bit, R. A., Che, K. H., Chung, C. W., Dittmann, A., Drewes, G., Drewry, D. H., Gordon, L., Grandi, P., Leveridge, M., Lindon, M., Michon, A. M., Molnar, J., Robson, S. C., Tomkinson, N. C., Kouzarides, T., Prinjha, R. K., and Humphreys, P. G. (2016) Discovery of I-BRD9, a Selective Cell Active Chemical Probe for Bromodomain Containing Protein 9 Inhibition. J. Med. Chem. 59, 1425.

(15) Fedorov, O., Castex, J., Tallant, C., Owen, D. R., Martin, S., Aldeghi, M., Monteiro, O., Filippakopoulos, P., Picaud, S., Trzepuzek, J. D., Gerstenberger, B. S., Bountra, C., Willmann, D., Wells, C., Philpott, M., Rogers, C., Biggin, P. C., Brennan, P. E., Bunnage, M. E., Schule, R., Gunther, T., Knapp, S., and Muller, S. (2015) Selective targeting of the BRG/MB1 bromodomains impairs embryonic and trophoblast stem cell maintenance. Sci. Adv. 1, e1500723.
Kitabayashi, I., Koseki, H., and Iwama, A. (2011) The Hbo1-Brd1/Brpf2 complex is responsible for global acetylation of H3K14 and required for fetal liver erythropoiesis. Blood 118, 2443–2453.

(30) Nyegaard, M., Severinsen, J. E., Als, T. D., Hedemand, A., Straarup, S., Nordentoft, M., McQuillin, A., Bass, N., Lawrence, J., Thirumalai, S., Pereira, A. C., Kandaswamy, R., Lydall, G. J., Sklar, P., Scolnick, E., Purcell, S., Curtis, D., Gurling, H. M., Mortensen, P. B., Mors, O., and Borglum, A. D. (2010) Support of association between BRD1 and both schizophrenia and bipolar affective disorder. Am. J. Med. Genet., Part B 153B, 582–591.

(31) Igoe, E., Fedorov, O., Tallant, C., Savitsky, P., Rogers, C., Owen, D., Deb, G., Somervaille, T., Andrews, D., Jones, N., Cheasty, A., Ryder, H., Brennan, P., Müller, S., Knapp, S., Fish, P., and Bayle, E. D. (2017) Design of a biased potent small molecule inhibitor of the bromodomain and PHD finger-containing (BRPF) proteins suitable for cellular and in vivo studies (2017). J. Med. Chem. 60, 668–680.

(32) Igoe, N., Bayle, E. D., Tallant, C., Fedorov, O., Meier, J. C., Savitsky, P., Rogers, C., Morias, Y., Scholze, S., Boyd, H., Canoosamy, D., Andrews, D. M., Cheasty, A., Brennan, P. E., Muller, S., Knapp, S., and Fish, P. V. (2017) Design of a chemical probe for the Bromodomain and Plant Homeodomain Finger-containing (BRPF) family of proteins (2017). J. Med. Chem. 60, 6998–7011.

(33) Philpott, M., Rogers, C. M., Yapp, C., Wells, C., Lambert, J. P., Strain-Damerell, C., Burgess-Brown, N. A., Gingras, A. C., Knapp, S., and Muller, S. (2014) Assessing cellular efficacy of bromodomain inhibitors using fluorescence recovery after photobleaching. Epigenet. Chromatin 7, 14.

(34) Tallant, C., Valentini, E., Fedorov, O., Overvoorde, L., Ferguson, F. M., Filippakopoulos, P., Svergun, D. L., Knapp, S., and Ciulli, A. (2015) Molecular basis of histone tail recognition by human TIP5 PHD finger and bromodomain of the chromatin remodeling complex NoRC. Structure 23, 80–92.

(35) Filippakopoulos, P., Picaud, S., Mangos, M., Keates, T., Lambert, J. P., Barsey-Lovejoy, D., Felletar, I., Volkmer, R., Muller, S., Pawson, T., Gingras, A. C., Arrowsmith, C. H., and Knapp, S. (2012) Histone recognition and large-scale structural analysis of the human bromodomain family. Cell 149, 214–231.

(36) Roux, K. J., Kim, D. L., and Burke, B. (2013) BioID: a screen for protein-protein interactions. Curr. Protoc Protein Sci. 74 (19), 23.

(37) Kitamura, T., Qian, B. Z., and Pollard, J. W. (2015) Immune cell promotion of metastasis. Nat. Rev. Immunol. 15, 73–86.

(38) Lamoureux, F., Baud'huin, M., Rodriguez Calleja, L., Jacques, C., Berreur, M., Redini, F., Lecanda, F., Bradner, J. E., Heymann, D., and Ory, B. (2014) Selective inhibition of BET bromodomain epigenetic signalling interferes with the bone-associated tumour vicious cycle. Nat. Commun. 5, 3511.

(39) Philpott, M., Yang, J., Tumber, T., Fedorov, O., Uttarkar, S., Filippakopoulos, P., Picaud, S., Keates, T., Felletar, I., Ciulli, A., Knapp, S., and Heightman, T. D. (2011) Bromodomain-peptide displacement assays for interactome mapping and inhibitor discovery. Mol. BioSyst. 7, 2899–2908.

(40) Yang, M., Birnbaum, M. J., MacKay, C. A., Mason-Savas, A., Thompson, B., and Odgren, P. R. (2008) Osteoclast stimulatory transmembrane protein (OC-STAMP), a novel protein induced by RANKL that promotes osteoclast differentiation. J. Cell. Physiol. 215, 497–505.