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Design and chemoproteomic functional characterization of a chemical probe targeted to bromodomains of BET family proteins†

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Bromodomain-containing proteins form the signal-reading element of a principal system for the control of gene expression in eukaryotes. Their potential as targets for selective drug action is increasingly being assessed and exploited. Deep characterization of the specificity, potency and other attributes of prototypical agents is an essential element of this process. Continuing studies of a dihydroquinazolinone-based series (prototype: **PFI-1**) with specificity for members of the BET (bromodomain and extra terminal) family led to the discovery of quinolin-2(1*H*)-one inhibitors with similar potency and selectivity, but increased chemical stability. Structure-guided design then led to the elaboration of a desthiobiotinylated analog retaining a high fraction of the potency of its parent compound and therefore suitable for chemoproteomic affinity capture experiments. These experiments, conducted using nuclear extracts of THP-1 cells, extended confidence in the selectivity of the series as first proposed. An additional and subsequent evaluation of specificity performed with a panel of recombinant bromodomains (BROMOscan™, DiscoveRx) supported the BET family specificity of the dihydroquinazolinone and quinolin-2(1*H*)-one series while adding appreciation of weaker effects shown at other bromodomains.

Introduction

In drug discovery research, a chemical tool is a compound that possesses the principal defining activity of a desired drug but lacks additional attributes that would permit its full development. Chemical tools are used to test the validity of discovery concepts at an early stage, helping with assessments of the attractiveness of particular targets and modalities in addition to uncovering potential liabilities and safety risks. Work done with these compounds can be influential. For example, there have been over 300 citations of the report[1] describing A-769662, a prototypical activator of AMP-activated protein kinase, despite this compound not possessing all of the attributes of a drug candidate. Although tool compounds interact with their intended targets at the molecular level and produce desirable physiological responses in preclinical tests, they have usually been excluded from clinical development. Despite this, they remain valued agents for investigative studies of the mechanisms that they engage.[2]

Another example of a chemical tool is **PFI-1** (**1**)¶ (Fig. 1), an agent that binds specifically to recombinant bromodomains (BRD) of proteins belonging to the bromo and extra terminal (BET) domain family.[3,4] BET family proteins contribute to the important function of reading epigenetic fingerprints that take the form of acetylated lysine residues on histone scaffolding proteins and affect chromatin structure and DNA accessibility. Thus, epigenetic changes effect alterations in gene expression that do not originate in changes in the DNA sequence. Once established, these chemical changes in DNA or histones can persist through many cell divisions, with specific patterns of gene expression determined by the epigenetic profile of the cell.

BRD are autonomously folded protein units that recognize and bind to ε-*N*-acetyl-lysyl residues of histones, forming the "reader" counterpart to the histone acetyltransferases that "write" signals related to the control of gene expression by

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¶ **PFI-1**, also known as PF-06405761, is commercially available from Sigma Aldrich (catalog # SML0352).

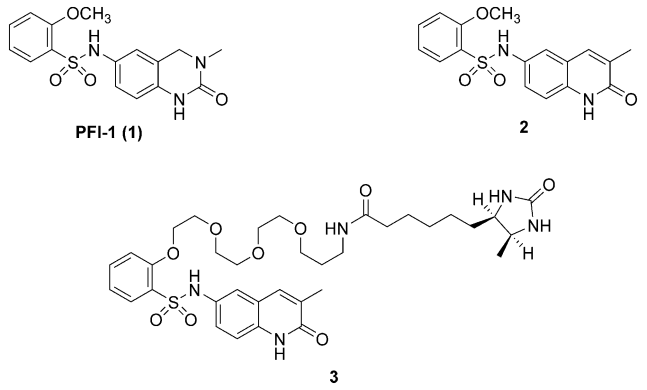


Fig. 1 Dihydroquinazolinone **PFI-1** and the alternative quinolin-2(1*H*)-one probe **2**, which lacks sensitivity to oxidative insertion of nucleophilic solvents. The desthiobiotinylated compound **3** was captured on streptavidin beads in the course of the affinity capture experiments.

N-acetylating histone tails at lysine side chains. For example, the BET family member BRD4 can recognize and bind to acetylated lysine residues on histones 3 and 4, allowing positive transcription elongation factor b (P-TEFb) to dock, phosphorylate RNA polymerase II, and facilitate efficient transcription of mRNA.[5] This has made BET family members, and in particular BRD4, attractive targets for therapeutic intervention aimed at directly modulating gene expression, a potentially powerful but presently underexploited mode of pharmaceutical action. Their potential significance as antitumor targets is at the leading edge of this effort.[4,6–9] Several medicinal chemistry efforts have now identified small molecule BET inhibitors that belong to different chemical series that represent alternative scaffolds for future drug discovery.[10]

Efforts to develop selective inhibitors of gene expression *via* targeted inhibition of BET family proteins (as opposed to broad antagonism of all BRD-containing proteins) are an endeavor that requires multiple well-characterized chemical tools. For example, access to a panel of chemical tools with diverse selectivity profiles within the BET subfamily of bromodomains would permit investigation of whether or not a specific BET family member (*e.g.* BRD4) can drive the transcription of a unique set of genes or, alternatively, that BET proteins exhibit overlapping and/or redundant roles. Likewise, in the context of a specific disease, the expression of BET proteins may be skewed to particular family members that significantly influence gene transcription. The ability to interrogate the respective roles of specific BET proteins in diseased cells with a set of well characterized chemical tools would yield important insights into pathological gene transcription. In the case of the prototype compound **PFI-1**, its potential has to some extent been examined through cell biological and structural biological approaches.[3,4] Here we evaluate the specificity of a close analog using the central chemoproteomic strategy of protein affinity capture.[11] We also provide additional biochemical characterization of the specificity of this compound using a proprietary panel of recombinant bromodomain targets (DiscoveRx BROMOscan™).

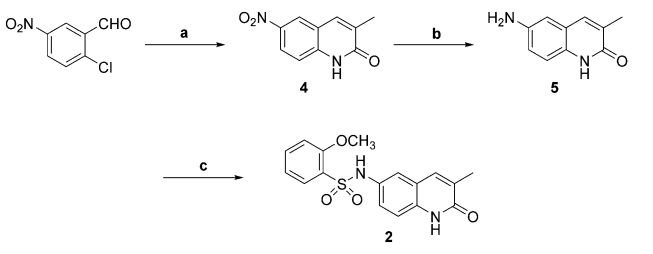
For reasons discussed below, **PFI-1** has been replaced by **2**,‖ a very close analog of **PFI-1** with increased chemical stability and equivalent or superior potency. A derivative of **2** has also been prepared which is suitable for affinity capture through a linked desthiobiotin moiety (**3**). Using nuclear extracts from cultured human cells that were shown proteomically to contain a significant fraction of known BRD-containing proteins, the novel probe was used to conduct protein affinity capture studies. Specificity was ensured by using **2** as a competitive agent, an approach complemented by the use of SILAC (stable isotope labeling with amino acids in cell culture) methodology in the proteomic analyses.[12]

Results and discussion

Subsequent to the original validation of **PFI-1** as a chemical probe with high selectivity for BET family proteins,[3,4] its dihydroquinazolinone ring system was found to be sensitive to oxidative insertion of nucleophilic solvents (see footnote 24 of Fish *et al.* (2012)[4]). In light of these data, we selected for the studies described below its aromatic quinolin-2(1*H*)-one analogue[13] **2** (Scheme 1). **2** is a chemically stable alternative to **PFI-1** which exhibits similar potency and selectivity (Table 1) against a panel of BRD. (In addition to the potencies measured by FP for **PFI-1**, **2** and **3** against BRD4-BD-1 and shown in Table 1, values of 115 nM, 105 nM and 83 nM, respectively, were measured by isothermal calorimetry.)

It was also important to confirm that **2** was active in a whole cell assay. In an LPS challenge assay in human peripheral blood mononuclear cells (PBMCs), **PFI-1** inhibited the release of interleukin-6 with an IC_{50} of 2830 nM. The quinolin-2(1*H*)-one analog **2** was a more potent inhibitor in the same assay, with an IC_{50} of 610 nM (Fig. 2). Its combination of superior stability and modestly improved bioactivity compared to **PFI-1** demonstrated its suitability for the present study. This investigation comprised a series of experiments in which chemoproteomic methodology was applied to characterizing the specificity of our quinolin-2(1*H*)-one probe against a wide sample of BRD-containing proteins from nuclear extracts of THP-1 cells.

The planned chemoproteomic studies required a biotinylated (or desthiobiotinylated) affinity capture probe that retained all or most of the affinity of **2** for BRD proteins,



Scheme 1

‖ Compound **2**, also known as PF-06482483, will be made commercially available from Sigma Aldrich.

Table 1 Relative affinities (by fluorescence polarization assay) of **PFI-1**, **2**, **3**, and reference compounds **IBET-762** and **IBET-151** for BET family bromodomains and CREBBP

	K_i (nM)				
Bromodomain	**PFI-1**	**2**	**3**	**IBET-151**	**IBET-762**
BRD4-BD1	88	27	43	17	58
BRD4-BD2	894	213	717	181	35
BRD2-BD2	122	102	321	217	83
BRD3-BD1	175	71	113	37	112
BRD3-BD2	294	207	146	118	48
BRDT-BD1	565	423	521	209	528
CREBBP	3800	2060	11 500	692	>14 500

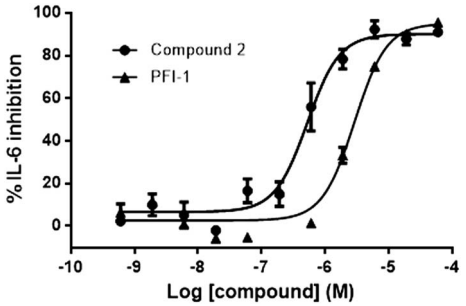


Fig. 2 The effects of compound **2** and **PFI-1** on LPS induced interleukin-6 (IL-6) release assayed in human PBMCs. IC_{50} values of 610 nm ($n = 4$) and 2830 nm ($n = 5$) were obtained for compound **2** and **PFI-1** respectively.

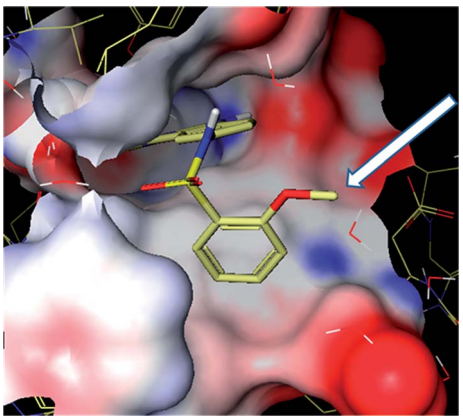


Fig. 3 Crystal structure of compound **PFI-1** bound to bromodomain 1 of BRD4 (PDB code 4e96). See also P. V. Fish *et al.*, *J. Med. Chem.*, 2012, **55**, 9831–9837. The structure was interpreted as suggesting that a linker group to facilitate immobilization through desthiobiotin could be placed at the ortho position of the aryl sulfonamide ring, from which the methoxy group of **PFI-1** points into solvent (arrow).

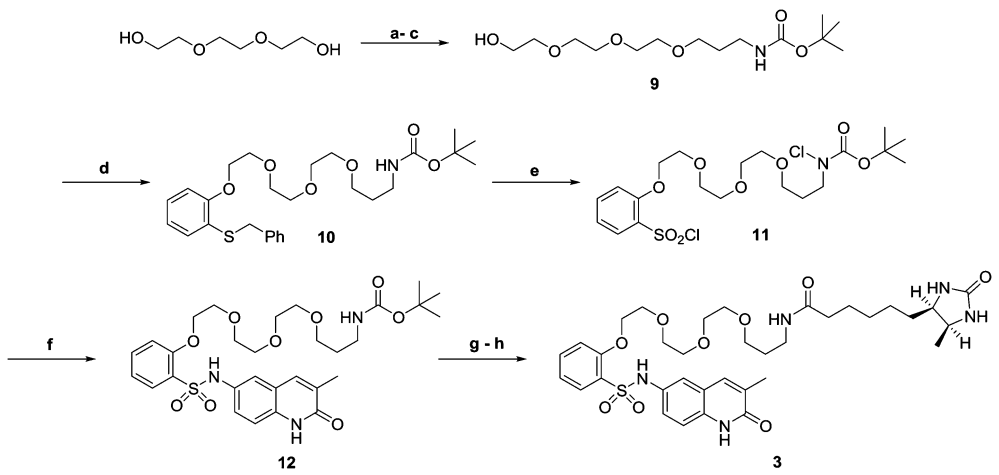
analogous to a previously described derivative of **IBET-762.**[9] Thus, it was essential to identify a region of molecule **2** which would tolerate homologation of an appropriate linker without interfering with binding to BRD proteins. Examination of the previously described[4] structure of **PFI-1** bound to the first bromodomain of BRD4 (Fig. 3) suggested that the *ortho*-position of the aryl sulfonamide pointed to solvent and could serve as an exit vector from the binding site that would not alter affinity to BRD targets. As this observation should equally apply to **2**, we devised the route shown in Scheme 2 to provide compound **3**. Compound **3** was assembled in a modular fashion, in which the active probe was coupled to desthiobiotin through a (3-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)propyl)amine linker. The linker, **9**, which was generated from triethylene glycol and acrylonitrile, followed by hydrogenation and Boc protection, afforded the amine handle to which desthiobiotin would be coupled. Alcohol, **9**, was combined with 2-(benzylthio)phenol under Mitsunobu conditions to yield the desired intermediate **10**. Conversion to the sulfonyl chloride (**11**) and coupling to the active probe (**5**) resulted in compound **12**. Removal of the BOC group, followed by standard amide coupling conditions with desthiobiotin resulted in the desired probe **3**.

Fluorescence polarization (FP) is a favored mode of assay for rating the respective affinities and selectivities of compounds that bind to bromodomains,[14,15] but it requires provision of a fluorescent probe against which test compounds can compete. In similar fashion to the preparation of **3**, we terminated the poly-alkoxy linker with Cy5 fluorescent dye (GE Healthcare, cat. PA15100: see preparation of **PF-411FP** in ESI†). The availability of **PF-411FP** then made it possible to assess the extent to which **3** retained the activity of **2**. In a FP-based assay of binding to BRD4-BD1 (Table 1), **2** and the pull-down probe **3** both exhibited slightly higher affinity for the protein target than **PFI-1**, similar potency to previously reported **IBET-762** (ref. 14 and 16) (currently in Phase 1 clinical trials), and slightly lower affinity when compared to **IBET-151.**[17]

In vitro BET family binding selectivity profiles of the compounds were then assessed by FP assay using six recombinant-expressed BET family bromodomains (Table 1, note: BRD2-BD1 and BRDT-BD2 proteins were not available at the time of this study). Our soluble competitor compound **2** and the affinity probe **3** demonstrated very similar potency and selectivity profiles against these six BET family proteins (Fig. 4), with both **2** and **3** showing 8–15× selectivity for the first bromodomain of BRD4 (BRD4-BD1) over BRD4-BD2 and BRDT-BD1.

The majority of BET ligands disclosed in the literature are either non-selective for the first bromodomain of BRD4 (BRD4-BD1) *versus* the second bromodomain of BRD4 (BRD4-BD2), or exhibit modest selectivity for BRD4-BD1 over BRD4-BD2. A notable exception is RVX-208 (currently in Phase 2 clinical trials for cardiovascular indications) which exhibits selectivity for BRD4-BD2.[18] Although the sequence homology between the first and second bromodomains of BRD4 is much lower than that between BRD4-BD1 and BRD2-BD1,[14,19] it is difficult to rationalize the observed selectivity for these compounds in the absence of crystallographic data of their binding modes with each of these proteins.

Also of note is the very similar BET family selectivity profile for both **2** and **3** when compared to our original probe molecule. Fig. 4 also highlights some additional differences in BET family selectivity for the set of molecules evaluated. **IBET-151** demonstrated a very similar BET family profile when compared to **2**



Scheme 2

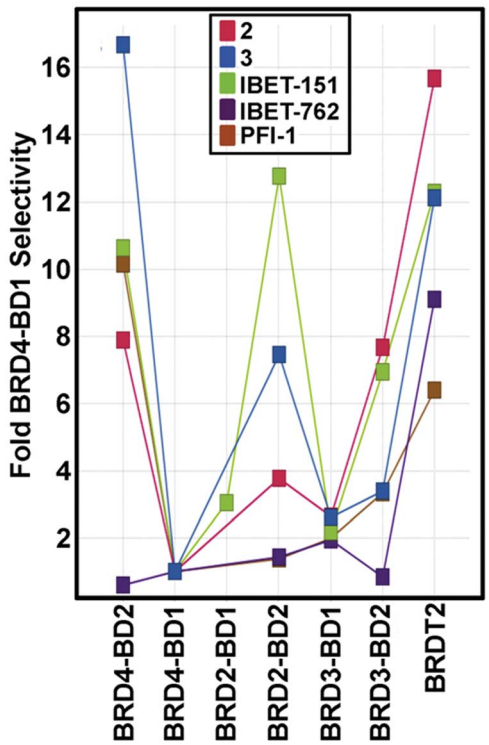


Fig. 4 BET family bromodomain selectivity profile of **PFI-1**, **2**, **3**, **IBET-762** and **IBET-151** as measured by the fluorescence polarization assay. Values on the vertical axis represent the ratio of each compound's K_d from a particular bromodomain to its K_d from BRD4-BD1: for example, the affinity of **3** for BRD4-BD2 is 17-fold weaker than its affinity for BRD4-BD1.

and **3**, but it has significantly less selectivity *versus* CREBBP. In contrast, **IBET-762** demonstrated similar potency against all of the BET domain proteins with the exception of BRDT for which it has comparable selectivity (~10×) when compared to **2** and **3**. Overall, the functional consequences of these different selectivity profiles have not yet been explored and require future investigation. These data support the view that **PFI-1** and compound **2** generally have comparable affinities for these BRD, and that **3** retains a substantial fraction of the affinity shown by **2** for each protein target with a very similar selectivity profile. In summary, the *in vitro* binding results appeared to qualify **3** as suitable for use in affinity capture studies in extracts from whole cells. Although the above results provide some insights into selectivity profiles for BET family proteins, the main objective of this work was to understand the extent to which our probe molecules exhibited selectivity against the broader BRD family in an authentic biological extract.

These data, which reinforce earlier information about the properties of **1** and **2**, opened the way for preliminary tests of the ability of **3** to capture BRD proteins from a biological milieu. Using SDS-polyacrylamide gel electrophoresis as a readout, it was shown that **3** immobilized on streptavidin-coated agarose beads could capture recombinant His-tagged BD1 of BRD4 from solution (Fig. 5). Soluble compound **2** prevented capture (Fig. 5A), demonstrating that the capture was a specific event based on affinity between protein and immobilized compound. Building on this result with recombinant protein, it was also shown by immunodetection on western blots that agarose beads bearing **3** could capture native BRD4 protein from lysates of either HeLa (cervical cancer immortalized cell line) or THP-1 (immortalized human monocytic cell line derived from a patient with acute monocytic leukemia) cells (Fig. 5B).

The above results left us ready for experiments in which **3** was used to "fish" by affinity capture for proteins in a biological extract, using SILAC labeling to allow specific and nonspecific protein binding to be distinguished.[12] Nuclear extracts of THP-1 cells made a proteomically suitable starting material for this work (see below), and THP-1 cells also appeared biologically pertinent in view of findings that (i) bromodomain inhibitor JQ1 inhibits growth of monocytic cells from acute myelogenous leukemia patients,[20] and (ii) BET inhibitors may have utility in the treatment of chronic inflammatory conditions in which monocytes and macrophages are prominent.[21] Thus, THP-1 cells were grown using SILAC conditions, with the "light" culture matched by "heavy" cells grown on medium containing [$^{13}C_6$,$^{15}N_2$]-L-lysine and [$^{13}C_6$,$^{15}N_4$]-L-arginine. Proteomic analysis of a lysate of heavy cells showed >97% incorporation of the

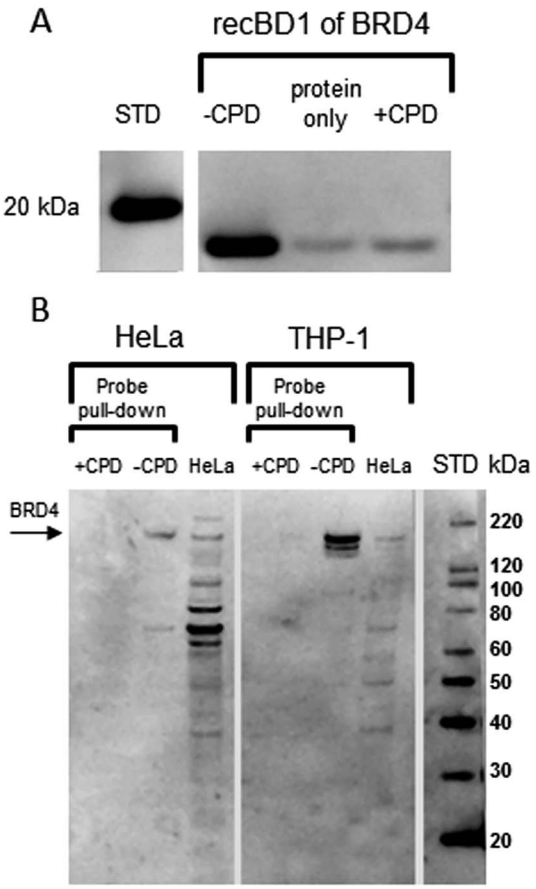


Fig. 5 Specific affinity capture of recombinant BRD4-BD1 or full-length BRD4 by compound **3**. (A) Western blot using anti-His-tag antibody. A 17 kDa His-tagged recombinant form of BD1 of BRD4 was captured by **3** linked to agarose beads (lane − cpd). Binding was prevented by competition with soluble compound **2** (lane + cpd). (B) Western blot showing affinity capture of BRD4 from HeLa or THP-1 cell lysates. Capture of BRD4 was specific, as indicated by the absence of capture in the presence of soluble compound **2**.

isotopically-substituted amino acids (data not shown), which was suitable for ratiometric proteomics.

As bromodomain-containing proteins are believed to be largely nuclear proteins,[7] nuclear extracts were prepared from the light and heavy THP-1 cells, and proteomic analysis was used to check the extent to which these extracts represented the nuclear fraction. The five highest-scoring hits in the sample (data not shown) were heterogeneous nuclear ribonucleoproteins A2/B1, DNA-dependent protein kinase catalytic subunit, heterogeneous nuclear ribonucleoprotein K, poly [ADP-ribose] polymerase 1, and nucleophosmin. Each of these proteins is located entirely or partly in the nucleus, according to the UniProtKB database, and none was among the top fifty hits in the cytoplasmic fraction prepared from the same lysate. This indicated that valid nuclear extracts had been prepared. The list of detected proteins (550 in all) was then scanned for BRD-containing proteins, and 15 such proteins were detected (Table S1†). We considered this an acceptable fraction of the 46 known[7] BRD-containing proteins encoded by the human genome; some of the proteins not detected are specific to cell types of other lineage (for example, BRDT which is testes-specific).[22] Also, because high relative abundance favors detection in proteomics, we reasoned that the list may not include every bromodomain-containing protein in the nuclear fraction and theoretically capable of being affinity captured. This possibility later emerged as a fact (see below).

Details of the method for affinity capture studies are given in ESI.† Briefly, probe **3** (3 μM) was incubated with THP-1 cell nuclear extract in the absence (SILAC light) or presence (SILAC heavy) of 10 μM **2**, which was added as a competitor of specific binding. After 3 h incubation, streptavidin-coated magnetic beads were added to capture **3** and proteins bound specifically to it. The two fractions of beads were combined after washing, and protein was eluted using SDS-polyacrylamide gel buffer.

Following SDS-polyacrylamide gel electrophoresis to fractionate the sample, captured proteins from the +/− compound-treated nuclear extracts were identified by LC-MS/MS. A total of 60 proteins were detected with a false-positive rate of 1%, among which 28 were identified with two or more unique peptides (Table 2) when the minimum Mascot[23] peptide score was set to the moderately stringent value of 30. The BET-family members BRD3, BRD4, and BRD2 were each present among the captured proteins, with 12, 8 and 4 unique peptides identified, respectively. As shown by the SILAC scores of zero for the BET-family members, their capture by **3** was completely inhibited by the soluble compound **2**, as demonstrated by the absence of the heavy partners of the detected peptide peaks. This was not the case for other proteins, for which SILAC ratios close to 0.5 indicated that the heavy and light nuclear extracts had not been mixed in perfectly equal proportion, but that nonspecific capture of proteins other than the BET family members was at a consistent level. However, we cannot exclude that failure to capture additional BRD-containing proteins is to some extent due to these proteins being complexed with protein binding partners and therefore inaccessible to the probe.

Subsequent to the chemoproteomic work, a broader survey of the bromodomain specificity of **PFI-1** and **3** was conducted using the DiscoveRx BROMOscan™ platform (Table S2† and Fig. 6). Consistent with earlier results, both **PFI-1** and **3** showed strong specificity for bromodomains of proteins from the BET family. Both exhibited >25 fold selectivity in favor of BET-family bromodomains as compared with all other bromodomains evaluated (Fig. 6), further supporting our chemoproteomics work demonstrating that the biotinylated probe had a similar selectivity profile to compound **PFI-1**. It was a point of interest that **3** exhibited higher potency than **PFI-1** against BRD4-BD1 in the BROMOscan™ study (7 nM *vs.* 62 nM), in contrast to the similar potency detected in both FP (43 nM *vs.* 88 nM) and ITC (83 nM *vs.* 115 nM). This difference is most likely attributable to differences in assay platforms (D. Treiber, DiscoveRx, personal communication). Overall, the data strongly supported the conclusion that among the set of bromodomains tested, **3** exhibits a minimum of about 25-fold specificity for those belonging to proteins of the BET family, and often a much greater selectivity.

Table 2 SILAC-based affinity capture of proteins from nuclear extract of THP-1 cells[a]

Accession	Description	# Unique peptides	Heavy/light
Q15059	Bromodomain-containing protein 3	12	0.00
P51659	Peroxisomal multifunctional enzyme type 2	12	0.52
O60885	Bromodomain-containing protein 4	8	0.00
P23246	Splicing factor, proline- and glutamine-rich	6	0.41
D6RAN4	60S ribosomal protein L9 (fragment)	6	0.82
P46783	40S ribosomal protein S10	5	0.40
Q08211	ATP-dependent RNA helicase A	4	2.21
P68104	Elongation factor 1-alpha 1	4	0.56
P62081	40S ribosomal protein S7	4	0.69
B4DLW8	Probable ATP-dependent RNA helicase DDX5	4	0.47
P25440	Bromodomain-containing protein 2	4	0.00
Q15233-2	Non-POU domain-containing octamer-binding protein	3	0.43
Q00839-2	Heterogeneous nuclear ribonucleoprotein U	3	0.51
H0YB39	Heterogeneous nuclear ribonucleoprotein H	3	0.31
M0R0F0	40S ribosomal protein S5 (fragment)	3	0.44
O95243-4	Isoform 4 of methyl-CpG-binding domain protein	3	0.73
F8VTQ5	Heterogeneous nuclear ribonucleoprotein A1	2	0.38
P22626-2	Heterogeneous nuclear ribonucleoproteins A2/B1	2	0.38
P60709	Actin, cytoplasmic 1	2	0.40
P62701	40S ribosomal protein S4, X isoform	2	0.65
P35637-2	Isoform short of RNA-binding protein FUS	2	0.52
P37108	Signal recognition particle 14 kDa protein	2	1.05
P62913-2	60S ribosomal protein L11	2	0.64
Q92841-1	Probable ATP-dependent RNA helicase DDX17	2	0.48
Q96PK6	RNA-binding protein 14	2	0.21
Q5T6W1	Heterogeneous nuclear ribonucleoprotein K	2	0.76
K7EQ02	DAZ-associated protein 1	2	0.68
Q09472	Histone acetyltransferase p300	2	0.47

[a] Limited to proteins with minimum of 2 peptides detected; hits on keratins and trypsin not shown.

According to the BROMOscan™ data (Table S2†), BRDT (bromodomain testis-specific protein, 10–20× selectivity) and BRPF1 (>100× selectivity) displayed moderate, but measurable interactions with compound **3**, but these proteins were not detected within the soluble nuclear fraction used in these sets of experiments. CREBBP (>300× selectivity) was found to be present in the soluble nuclear fraction, albeit in low abundance. Failure to detect it in the affinity capture experiment may therefore confirm the weak binding affinity between this protein and compound **3** as identified by BROMOscan™ and in-house binding data (Table 1 and S2†). Although it is possible that the linker group in **3** could interfere with its binding to some bromodomain family members, the lack of differentiation between the selectivities of **PFI-1** *versus* **3** in the BROMOscan™ study (Fig. 6 and Table S2†), and the conserved acetyl lysine binding pocket of the BRD family suggests this is unlikely.

This work highlights the combined use of SILAC technology with a biotinylated small molecule probe to assess the selectivity profile of an unlabeled compound for a particular class of intracellular target proteins. This technique provides data

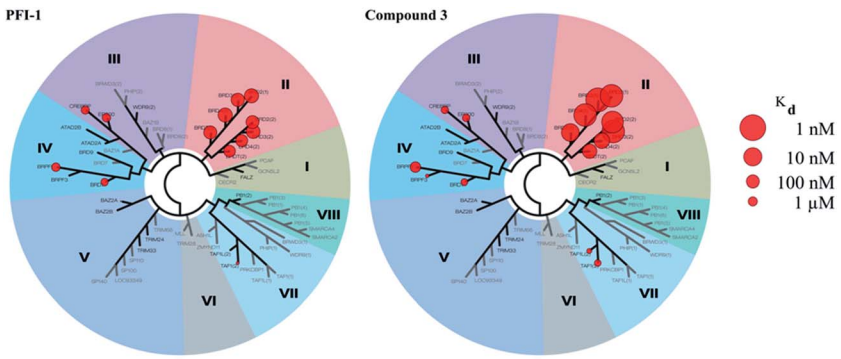


Fig. 6 TREEspot™ plots of BROMOscan™ data for **PFI-1** and compound **3**. images generated using TREEspot™ software tool and reprinted with permission from KINOMEscan®, a division of DiscoveRx corporation, © DiscoveRx corporation 2010.

based on the capture of authentic cellular proteins that complement results from biochemical screening platforms that use recombinant proteins. In this instance our study was not quantitative, and dose response studies with the probe and soluble competitor would be required to gauge selectivity between the different BET family members.

Our efforts in probe design have demonstrated that adoption of a polyglycol spacer-bearing desthiobiotinylated derivative, **3**, does not sacrifice affinity for any of the bromodomains which have been assessed to date. In addition, we have demonstrated a somewhat different binding/selectivity profile of compounds **2** and **3** in THP1 nuclear extracts compared to that described for **IBET-762** and **IBET-151** in HL60 nuclear extracts.[9] Our fluorescent polarization assay revealed enhanced selectivity for **2** over CREBBP compared to that observed for **IBET-151**. This selectivity profile may have significant impact on gene transcription because (i) CREBBP is known to have intrinsic histone acetyltransferase activity and (ii) it acts as a scaffold to stabilize additional protein interactions within the transcription complex. For example, CREBBP interacts[24,25] with a diverse group of transcription factors including CREB, p53, NF-κB and AP-1, which can lead to augmentation of gene transcription. Thus, in the seminal paper by Dawson *et al.*,[9] wherein they report on **IBET-151** *in vitro* potency and *in vivo* efficacy, concentrations above 1 μM would be expected to also interact with CREBBP and affect gene transcription. Consequently, in their studies that addressed *in vivo* efficacy in MLL leukemia models, **IBET-151** was dosed at 30 mg kg^{-1} intraperitoneally once daily. From the PK analysis of this compound reported in their paper,[9] systemic exposures would be expected to exceed 1 μM for a significant portion of the day, and this leads us to question whether a proportion of the observed *in vivo* pharmacology is due to inhibition of CREBBP in addition to the expected BET family pharmacology. Of note, Delvecchio *et al.*[26] recently published the crystal structure of the p300 catalytic core, a closely related HAT enzyme with structural and functional similarities to CREBBP. This compact module consists of the bromodomain together with PHD, RING and HAT domains, and tight integration of the chromatin substrate-binding domains into the enzymatic core of the acetyltransferase explains why substrate recognition is coupled to HAT activity and why mutation of these domains can lead to aberrant HAT function and pathogenesis. These observations provide further validation that binding to the bromodomain of CREBBP will impact gene transcription even in the absence of BET activity and highlight the risk that a portion of **IBET-151** efficacy is driven by off-target effects on CREBBP.

Interestingly, we were unable to detect BRD9 in the nuclear extracts of THP-1 cells. Although this may simply reflect limitations in the sensitivity of our proteomic analysis, we were also unable to show binding of **2** to BRD9 in a DiscoveRx BROMOscan™. In contrast, Dawson *et al.*[9] reported that both **IBET-762** and **IBET-151** could block the capture of BRD9 by acetylated histone tail peptides in a nuclear extract derived from HL60 cells. Similarly, in proteomic profiling studies in which they used a set of biotinylated histone peptides immobilized to streptavidin-coated beads to capture binding proteins from HL60 nuclear extracts, they demonstrated that both **IBET-151** and **IBET-762** inhibited binding of BRD9. They speculated that BRD9 was not a direct target of the IBET molecules and that it was subject to competition because it can form complexes with BRD4. At least in HL60 nuclear extracts this appears to be the case, as they demonstrated pull down with BRD4 in immunoprecipitation studies. However, it is intriguing that we were unable to detect BRD9 in THP1 cells, despite this cell line's ability to produce IL-6 upon LPS stimulation at similar levels to that observed in human whole blood. This leads us to question whether or not BRD9 is required for the transcription of IL-6 in certain cell types.

Conclusion

In summary, this work describes the design and utility of an affinity based probe coupled with a protein capture method to quantify the selectivity of soluble competitor compounds for the bromodomain containing family of proteins identified in THP-1 cells. These results support the hypothesis that **3** is highly selective for BET family bromodomains in nuclear extracts of THP-1 cells and these results were confirmed when assessed in the proprietary BROMOscan™ panel of bromodomains. Further analysis using extracts from different cellular compartments and different cell types would provide additional information on the selectivity profile of soluble inhibitors of BRD proteins. Additionally, we have demonstrated a different bromodomain selectivity profile for **PFI-1**, **2**, and **3** compared to other BET inhibitors which could be exploited in future studies to explore the role of specific family members in gene transcription.

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