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Evaluation of Functional Groups as Acetyl-Lysine Mimetics for BET Bromodomain Inhibition

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Abstract
The ability of various functional groups to engage the acetyl-lysine (KAc) binding site within bromo- and extra-terminal domain (BET) protein family members BRD2, BRD3 and BRD4 was evaluated by screening small molecular fragments - coupled to a known arylsulfonamide scaffold - in biochemical inhibition assays. Useful structure activity relationships have been established and novel functional groups that bind to the KAc binding pocket identified. Additional microsomal degradation studies were also undertaken revealing significant differences in metabolic stability between two commonly employed BET inhibitor fragments.

1. Introduction
The bromo- and extra-terminal domain (BET) proteins BRD2, BRD3, BRD4 and BRDT are a class of epigenetic ‘readers’ that recognize ε-N-acetyl-lysine (KAc) residues.1,2,3 The binding interaction between BETs and acetyl-lysine residues of histone tails creates a scaffold for the assembly of protein complexes and can alter chromatin accessibility, ultimately leading to regulation of gene transcription and/or chromatin remodeling.4,5 A number of regulatory pathways involving specific BETs have been uncovered. For example, BRD2 disruption has been shown to cause obesity in mice,6 BRD3 is involved in erythroid maturation,7 BRD4 promotes the transcription of oncogenes such as c-MYC,8 and BRDT is essential for chromatin remodeling during spermatogenesis.9 Therefore, the inhibition of BET-chromatin binding may have a variety of biological consequences and potential therapeutic opportunities for BET inhibitors include use in neurological diseases, as antiviral therapy, as anticancer agents and for treatment of inflammatory and autoimmune diseases.10,11,12 In order to develop a more detailed understanding of the individual roles of BETs and fully realize the therapeutic potential of BET inhibitors, compounds displaying different BET selectivity profiles may be required.13,14,15

To date, a number of small molecule inhibitors of BETs have been reported in the academic and patent literature.16 Representative compounds include JQ1 (1),9 IBET-151 (2),17 PFI-1 (3),18 IBET-762 (4),19 5,20 and 621 (Figure 1). X-ray crystallographic data and competition assays demonstrate that BET inhibitors bind within the KAc binding pocket often via a specific KAc mimicking motif (highlighted in red for the abovementioned compounds in Figure 1).22 Despite the plethora of structural data available (more than 100 X-ray crystal structures for BET bromodomains can be found on the Protein Data Bank) the strategic, structure-based design of selective BET inhibitors remains challenging due to the high sequence homology between the KAc binding pockets of the family members.23 BET proteins contain two distinct acetyl-lysine binding sites, each of which is located within one of the two N-terminal bromodomains – termed domain 1 (D1) and domain 2 (D2). The bromodomains are a four α-helical bundle consisting of approximately 150 amino acids
which interact with acetylated lysine via conserved asparagine and tyrosine residues, the latter occurring through a network of highly structured water molecules. The KAc mimetic motifs of current BET inhibitors can be broadly classified by chemotype; either 1,2,4-triazoles, such as JQ1 and IBET-762, quinolones such PFI-1, dimethylisoxazoles such as IBET-151 and 5, or thiazolidinones such as 6.

![Figure 1](image_url)  
**Figure 1.** Representative BET inhibitors. KAc mimicking motif highlighted in red.

Notably, the 3,5-dimethylisoxazole is a structural motif found in a number of reported BET inhibitors such as IBET-151 and 5. In both of these compounds the dimethylisoxazole fragment acts as the KAc mimic, which is able to interact with the bromodomain asparagine residue (Asn140 in BRD4 D1) and a structured water molecule via a hydrogen bond acceptor oxygen and nitrogen atom respectively (Figure 2). The KAc mimicking capability of 3,5-dimethylisoxazoles was discovered following a fragment screen against BETs, which identified 3,5-dimethyl-4-phenylisoxazole as the fragment hit. Subsequent structure-guided optimization by substitution of the phenyl portion of the fragment hit led to the development of the 5, I-BET151 (2) and other compounds; however, the effects of modifying the dimethylisoxazole (KAc mimetic) portion of the fragment hit on binding affinity were not studied in detail.

In this work, we sought to understand the structure activity relationship (SAR) surrounding the heterocyclic KAc mimetic portion of such inhibitors. At the outset of our investigations a general SAR examination of the KAc binding motif within small molecules had not been reported though recently an in silico-driven analysis of acetyl-lysine mimetics was undertaken by Vidler et al, which identified 4 novel bromodomain-binding heterocycles. The aim of our work has been to synthesize compounds containing a diverse variety of functional groups appended to a scaffold with validated affinity for the BET proteins and to compare their ability to interact with KAc binding pockets of BRD2, BRD3 and BRD4 with the view to identify novel KAc mimetics. We chose compound 5 as a starting point for our investigations because 1) its mode of binding to the KAc binding pocket is well understood based on reported crystallographic data and 2) it provides a simple and readily diversifiable platform from which to explore independent changes to the acetyl-lysine mimetic portion while consistently maintaining the same arylsulfonamide scaffold. Although some specific features of the acetyl-lysine mimicking group were marked for
investigation, such as the nature and number of heteroatoms, presence of methyl groups and ring size etc., surveying broader chemical space also seemed appropriate given the deficit of such data available in the literature. Consequently, 5- and 6-membered heterocycles were the main focus of the SAR studies however other functional groups that may potentially interact with the acetyl-lysine binding site via hydrogen bonds, such as acetamides and sulfonamides were also investigated.

![Figure 2. Schematic representation of key KAc mimetic binding interaction of 5 with BRD4(D1) and focus of SAR investigations on the KAc mimicking group, Fg.](image)

2. Results and Discussion

2.1 Chemistry

The requisite *N*-cyclopentylphenylsulfonamide scaffolds were prepared using the general strategy outlined in Scheme 1. Thus, mono-protected cyclopentylamine (or the parent compound for 10) was coupled with commercially available arylsulfonyl chlorides, affording the corresponding sulfonamides 7a, 7b, 8-10.

![Scheme 1. Reagents and conditions: a) Et$_3$N, CH$_2$Cl$_2$, rt, 24 h; DMB = 2,4-dimethoxybenzyl.](image)

It was envisaged that diverse aryl and heteroaryl functional groups could be incorporated onto aryl bromides 7a and 7b by employing a Suzuki-Miyaura cross-coupling reaction. After considerable optimisation, a general method for enabling the Suzuki-Miyaura cross-coupling between 7a and 7b and a range of aryl boronic acids and esters, in a parallel fashion, was established using the PEPPSI-SiPr™ pre-catalyst with a dioxane:H$_2$O:DMF solvent system, in the presence of Cs$_2$CO$_3$ and under microwave irradiation. The products obtained from these reactions were subsequently treated with trifluoroacetic acid (TFA) to remove the 2,4-
dimethoxybenzyl (DMB) protecting group to furnish a collection of aryl- and heteroaryl-functionalized compounds (5 and 11-27, Scheme 2).\textsuperscript{31}

\begin{scheme}
\textbf{Scheme 2.} \textit{Reagents and conditions:} a) i) PEPPSI-SIPr\textsuperscript{TM}, Cs\textsubscript{2}CO\textsubscript{3}, dioxane, H\textsubscript{2}O, DMF, 90 °C, microwave, 12 h. ii) TFA, CH\textsubscript{2}Cl\textsubscript{2}, rt, 2 h.

Attempts to prepare 2-amino-6-methylpyridine-5-boronic acid or the corresponding pinacol ester for deployment in a Suzuki-Miyaura cross-coupling with 7a failed. Instead, the boronic ester 28 was prepared and the Suzuki-Miyaura cross-coupling reaction with 2-amino-4-bromo-6-methylpyridine proceeded smoothly to afford, after deprotection, aminopyridine derivative 29 (Scheme 3). Sonogashira cross-coupling between bromide 7a and triethylsilylacetylene gave alkyne 30. Removal of the triethylsilyl group provided the terminal alkyne 31, which underwent copper-catalysed [3+2] cycloaddition with trimethylsilylazole affording mono-substituted triazole 32. Subsequent alkylation of triazole 32 with iodomethane resulted in the formation of a mixture of regioisomeric N-methyltriazoles 33 and 34 (3:2 ratio respectively), which were separated chromatographically and characterized using 2D-NMR experiments and comparison with literature data for other methylated 1,2,3-triazoles (see supporting information). Subsequent acidic deprotection of 33 and 34 gave isomeric methyltriazoles 35 and 36 respectively. Additionally, pyridines 19a and 29 were converted into the corresponding N-oxides by treatment with \textit{m}-CPBA providing 37 and 38 respectively (Scheme 3).
Scheme 3. Reagents and conditions: a) B\textsubscript{2}pin\textsubscript{2}, Pd(dppf)Cl\textsubscript{2}, PPh\textsubscript{3}, KOAc, DMSO, 100 °C, 12 h. b) i) 2-aminomethyl-5-bromo-6-methylpyridine, PEPPSI-SIPr\textsuperscript{TM}, Cs\textsubscript{2}CO\textsubscript{3}, dioxane, H\textsubscript{2}O, DMF, 90 °C, microwave, 12 h. ii) TFA, CH\textsubscript{2}Cl\textsubscript{2}, rt, 2 h. c) Triethylsilylacetylene, Pd(PPh\textsubscript{3})\textsubscript{4}, CuI, Et\textsubscript{3}N, THF, 80 °C, 12 h. d) TBAF, THF, 2 h, 0 °C. e) Trimethylsilylazide, CuI, DMF, MeOH, 90 °C, 12 h. f) MeI, NaHCO\textsubscript{3}, DMSO, rt, 12 h. g) TFA, CH\textsubscript{2}Cl\textsubscript{2}, rt, 2 h. h) m-CPBA, CH\textsubscript{2}Cl\textsubscript{2}, rt, 5 h; DMB = 2,4-dimethoxybenzyl.

Nitrile 8 was converted into methyloxadiazole 39 via a reaction with dimethylacetamide-dimethylacetal and hydroxylamine followed by subsequent acid-promoted deprotection. Thermal [3+2]-cycloaddition reaction of 8 with sodium azide afforded tetrazole 40, alkylation of which with iodomethane gave an inseparable mixture of 1- and 2-N-methyl regioisomers in an approximate 1:1 ratio. Removal of the DMB group under acidic conditions allowed for the chromatographic separation of the regioisomeric products 41 and 42 and the identity of each regioisomer was established using NMR experiments (see supporting information). Nitrile 8 was also converted into the acetamide 43 in a three-step sequence involving reduction of the nitrile, acetylation of the resultant primary amine and N-DMB cleavage (Scheme 4).
Scheme 4. Reagents and conditions: a) i) dimethylacetamide-dimethylacetal, NH₂OH.HCl. ii) HCl, dioxane, 2 h, rt. b) i) MeI, NaHCO₃, DMSO, rt, 12 h. ii) HCl, dioxane, rt, 12 h. c) Na₂N₃, DMF. d) i) H₂, Pd/C, MeOH, ‘H-cube™’ flow reactor. ii) Ac₂O, EtOAc, HCl. iii) HCl, dioxane, rt, 2 h.

The nitro arylsulfonamide 10 was hydrogenated to the corresponding aniline 44 in the presence of Pd/C under flow conditions and converted into either the acetamide 44 or the methanesulfonamide 45 using standard protocols (Scheme 5).

Scheme 5. Reagents and conditions: a) H₂, Pd/C, 40 °C, ‘H-cube™’ flow reactor. b) i) Ac₂O, EtOAc, HCl, rt, 2 h. ii) TFA, CH₂Cl₂, rt, 2 h. c) i) methanesulfonyl chloride, pyridine, CH₂Cl₂, 0 °C, 2h. ii) TFA, CH₂Cl₂, rt, 2h.

Reduction of nitroarene 10 and subsequent condensation of the resultant aniline (47) with acetylhydrazine and the dimethylacetals of either dimethylformamide or dimethylacetamide provided either 3-methyl- or 3,5-dimethyl-1,2,4-triazoles (48 or 49) respectively (Scheme 6).

2.2 Biochemical Assays
An ALPHAscreen\(^{TM}\) assay was chosen for evaluation of the KAc mimetic groups as it allows for identification of functional groups that bind to the acetyl-lysine binding site of BRD2(D1), BRD3(D1) and BRD4(D1) through competition with an acetylated histone peptide.\(^{32}\) Only single bromodomains (D1) of the full-length protein (which each contain two bromodomains) were used in the assay to avoid multiple combinations of binding peptide and inhibitor, which may complicate interpretation of the results. Further, previous studies have shown that inhibition of the N-terminal bromodomain of BRD4 (BRD4(D1)) is more effective than inhibition of the C-terminal bromodomain (BRD4(D2)) for eliciting biological response.\(^{13}\) Although the binding affinity of these compounds was expected to be low (IC\(_{50}\) in the µM range), we rationalized that the compounds could be employed in relatively high concentration (5-10 µM) because their low molecular weight would reduce the possibility of assay interference through precipitation or other mechanisms. This approach therefore should enable a rapid and reliable assessment of the acetyl-lysine mimicking capability of small pharmacophore fragments.

The results of the primary screening are presented in Table 1 (full data in Table S1, Supplementary Information) which show that the previously described dimethylisoxazoles 11a and 5 were the most active compounds on all BET bromodomains examined, independent of the nature of the para-disposed group (R = Me or MeO) although this had a moderate impact on binding affinity, the selectivity consistently favoured BRD3 > BRD4 > BRD2. The structurally similar 3,5-dimethylpyrazole 12a was also quite potent against BRD3 and BRD4 and showed a weaker affinity for BRD2 than observed for the isoxazoles. The 1,3,5-trimethylpyrazole 13a displayed no detectable inhibition which is presumably a consequence of the N-methyl disrupting important H-bonding interactions with the asparagine or tyrosine residues (Asn140 or Tyr97 respectively in BRD4(D1)). Similarly, pyrazole 14a, which contains no methyl groups on the KAc mimic, was completely inactive, underscoring the importance of methyl groups in mimicking the native acetyl-lysine. Pyrazole 15a on the other hand, showed some inhibition albeit weak, despite the presence of only a single methyl group and a single H-bond acceptor on the KAc mimetic but again had a similar BET binding selectivity profile to the isoxazoles. Thiophenes 16a and 17a, pyridines 18a-20a, pyrimidine 21a, phenylacetamides 22a and 23a, methylanilines 24a-26a or phenylsulfonamide 27a all showed little or no detectable binding at the concentration tested. However, pyridine 29 did exhibit moderate binding, possibly due to the addition of the o-methyl substituent (c.f. pyridine 20a) again highlighting the importance of the methyl group for the observed effect. The methyl-1,2,3-triazole 35 displayed binding affinity for BRD4 and significant selectivity over BRD2 and BRD3 in contrast to all other compounds examined. Interestingly, the isomeric triazole 36 displayed no detectable binding. Pyridine-N-oxide 37
was completely inactive, however, the N-oxide 38 (derived from pyridine 29) showed moderate binding affinity for BRD3 and BRD4. Oxadiazole 39 and tetrazole 41 showed no activity nor did tetrazole 42. Acetamides 43 and 45 showed very weak binding, as did the sulfonamide 46. Unexpectedly, 1,2,4-triazoles 48 and 49, which contain the KAc mimetic from known BET inhibitors JQ1 and IBET-762, showed very little detectable inhibition.
Table 1. ALPHAscreen™ binding data expressed as % of control (DMSO) at 5 µM compound concentration.

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Legend: 100% Full inhibition, 50% Partial inhibition, 0% No inhibition.
To confirm and quantify the single point ALPHAscreen™ assay data, the IC50s against BRD3 and BRD4 of 7 selected compounds were determined. Consistent with the single point data and with previously reported data20 the isoxazole 11a displayed IC50s of 0.20 µM and 1.2 µM against BRD3 and BRD4 respectively and 5 had IC50s of 0.25 µM and 0.67 µM. Dimethylpyrazole 12a showed slight selectivity for BRD3 (IC50 = 1.32 µM) over BRD4 (2.89 µM) while monomethylpyrazole 15a showed some selectivity for BRD3 (IC50 = 1.71 µM) over BRD4 (10.12 µM). In contrast to these compounds and consistent with the single point data, 1,2,3-triazole 35 displayed a slightly greater selectivity for BRD4 (IC50 = 4 µM) over BRD3 (IC50 = 10 µM). Finally, the IC50 data also confirmed the surprising lack of binding exhibited by 1,2,4-triazoles 48 and 49 (IC50s of each for BRD3 and BRD4 are ≥ 10 µM). Given the structural similarity between dimethylisoxazoles 11a and 5, and 1,2,4-triazoles 48 and 49 the molecular basis for the differences in their binding affinity to BETs remains unclear. Indeed, based on predicted H-bonding strength (pKHX) one would predict the triazole to have improved binding affinity,33 possibly indicating that a combination of stereoelectronic and orientational factors contribute to binding affinity in this instance. Accordingly, X-ray crystallographic and molecular modelling studies of such KAc mimetics are currently being pursued in our laboratories.

![Dose-response data for BRD3 and BRD4](image1)

**Figure 3.** Selected ALPHAscreen™ binding data IC50 for assays on BRD4(D1) and BRD3(D1).

### 2.3 Metabolism Studies

Given the ubiquitous deployment of 2,5-dimethyl-3,4-isoxazole containing compound IBET-151 and 1,2,4-triazole containing compounds JQ1 and IBET-762 for in vivo studies8,17,34 we sought to compare the metabolic stability of the corresponding KAc mimetic functional groups. Thus, IBET-151, JQ1, 5 and 49 were incubated with mouse, rat and human liver microsomes in order to assess their metabolic stability (Table 2).
Table 2. Summary of human, rat and mouse microsomal stability of IBET-151, JQ1, 5 and 49. *No measurable concentrations were detected past 5 minutes, therefore degradation parameters were estimated using the initial two time points (i.e. 2 and 5 minutes) only, hence values reported are approximate only.

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<th>Compound</th>
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<th>In vitro $\text{CL}_{\text{int}}$ (µL/min/mg protein)</th>
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</tbody>
</table>

Comparison of the metabolic stability of 5 and 49 demonstrated that 5 is degraded rapidly by microsomes from all three species ($t_{1/2}$ c.a. 5 min) while 49 is significantly more stable ($t_{1/2}$ c.a. > 1.5 h). Analysis of the major degradation metabolites of 5 revealed that $N$-dealkylation corresponding to loss of the cyclopentyl group [M-68] was an important degradation pathway that was not observed for triazole 49. In this instance therefore, the presence of the dimethylisoxazole moiety is a metabolic liability in comparison to the analogous 1,2,4-triazole group. In contrast, however, the dimethylisoxazole containing compound IBET-151 (2) was significantly more stable ($t_{1/2}$ > 2 h) than other compounds assessed in this work, while the 1,2,4-triazole containing compound (+)-JQ1 (1), which is commonly employed in in vivo studies, was degraded rapidly ($t_{1/2}$ c.a. 10 min), consistent with published data.$^{17,35}$ These data suggest that the heterocyclic KAc mimic moieties within these compounds are not the major site at which metabolic degradation takes place though the more lipophilic isoxazole moiety$^{36}$ may detrimentally impact metabolic stability.

3. Conclusion

A wide range of functional groups was evaluated for BET bromodomain inhibition using an ALPHAscreen$^{TM}$ assay. Among the functional groups screened were dimethylisoxazoles and 1,2,4-triazoles, which are common structural motifs in BET inhibitors. Previously described isoxazoles were the most potent inhibitors screened, however, 49 showed poor metabolic stability. By contrast, 1,2,4-triazoles, which share similarities with well-known inhibitors such as JQ1 and IBET-762, were less effective KAc mimetics than some pyrazoles and approximately equipotent with a 1,2,3-triazole and a 2-amino-6-methylpyridine, on this scaffold. This striking difference in binding affinity suggests that alternatives to 1,2,4-triazoles as KAc mimetics should be considered when optimising BET inhibitors. Additional microsomal stability studies demonstrated that some BET inhibitors have significant metabolic liabilities, which may have important implications for in vivo studies including clinical trials. The work described herein highlights the potential of expanding the chemical
space of acetyl-lysine mimetics in an effort to develop more potent, selective, and ultimately efficacious, BET inhibitors.

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Notes and references

13. S. Picaud, C. Wells, I. Felletar, D. Brotherton, S. Martin, P. Savitsky, B. Diez-Dacal,


22 PDB codes PFI-1 (4E96), JQ1 (3MXF), IBET-151 (4ALG), IBET-762 (2YEK), compound 5 (4HXL cyclohexyl derivative), compound 6 (no PDB code, see ref 19 for crystal structures of related derivatives).

28 For example [PDB code 4HXL for a cyclohexyl derivative] also see reference 18.

29 For example, Bamborough *et al.* (reference 18) showed that both 5 and the corresponding congener where the 2-methyl group was substituted for a methoxy group, both had moderate binding affinity (0.6 and 2.2 µM in TR-FRET assay respectively against BRD4).


31 In cases where a tert-butoxycarbonyl group was present on the boronic acid or ester coupling partners, it was cleaved under the conditions of the Suzuki-Miyaura cross-coupling reaction.

32 The tetra-acetylated peptide used in the ALPHAscreen assays in this work has similar binding affinity for BRD4(D1) and BRD2(D1) of 2.8 µM and 3.7 µM respectively (comparative data for BRD3(D1) obtained in the same assay is not available but is expected to be similar based on sequence homology). See reference 22.


36 E.g. 3,5-dimethyl-4-phenylisoxazole LogP = 2.32 vs 3,5-dimethyl-4-phenyl-1H-1,2,4-triazole LogP = 0.51; MarvinSketch Calculator Plugins were used for structure property prediction, Marvin 6.0.6, 2013, ChemAxon ([http://www.chemaxon.com](http://www.chemaxon.com))
This work provides new insights into a range of acetyl-lysine mimetics as BET bromodomain inhibitors.