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 **6**[21] (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.[24] 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**.

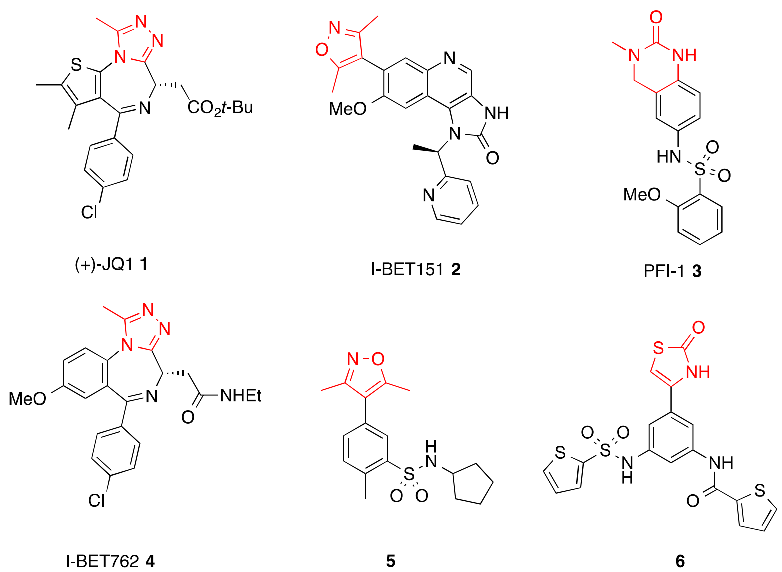

Figure 1. Representative BET inhibitors. KAc mimicking motif highlighted in red.

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 mimetic, 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.[25] 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.[25, 26] 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.[27] 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[28] 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.[29] 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.

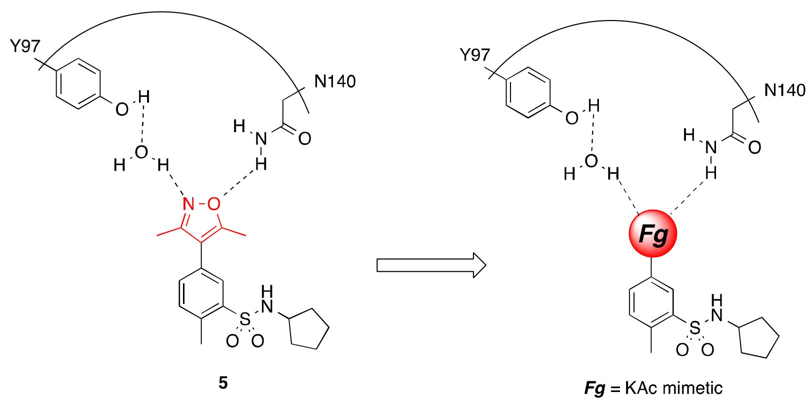

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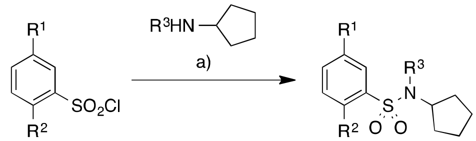
Figure 2. Schematic representation of key KAc mimetic binding interaction of **5** with BRD4(D1) and focus of SAR investigations on the KAc mimicking group, **Fg**.

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**.

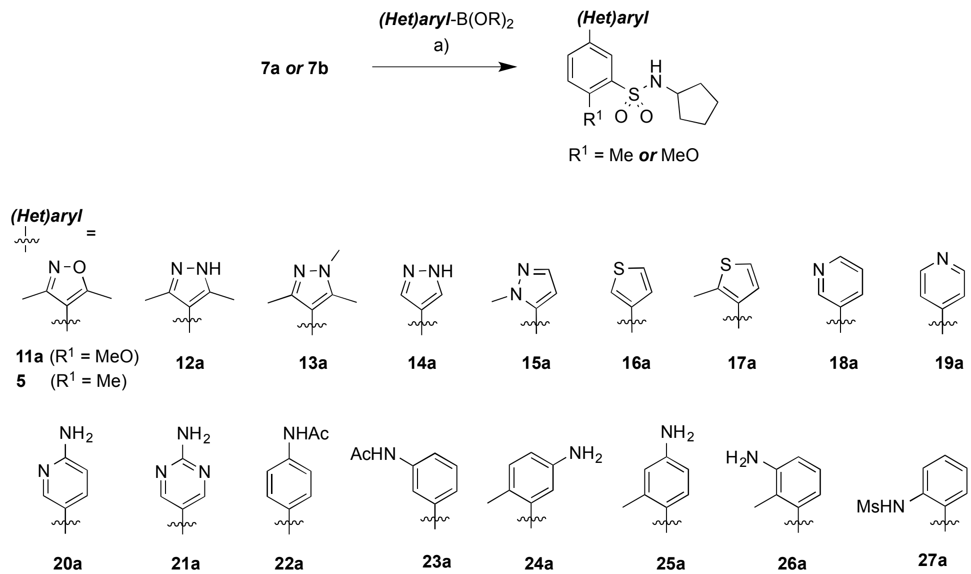


7a R^1 = Br, R^2 = MeO, R^3 = DMB
7b R^1 = Br, R^2 = Me, R^3 = DMB
8 R^1 = CN, R^2 = Me, R^3 = DMB
9 R^1 = NO_2, R^2 = Me, R^3 = DMB
10 R^1 = NO_2, R^2 = Me, R^3 = H

Scheme 1. *Reagents and conditions:* a) Et_3N, CH_2Cl_2, rt, 24 h; DMB = 2,4-dimethoxybenzyl.

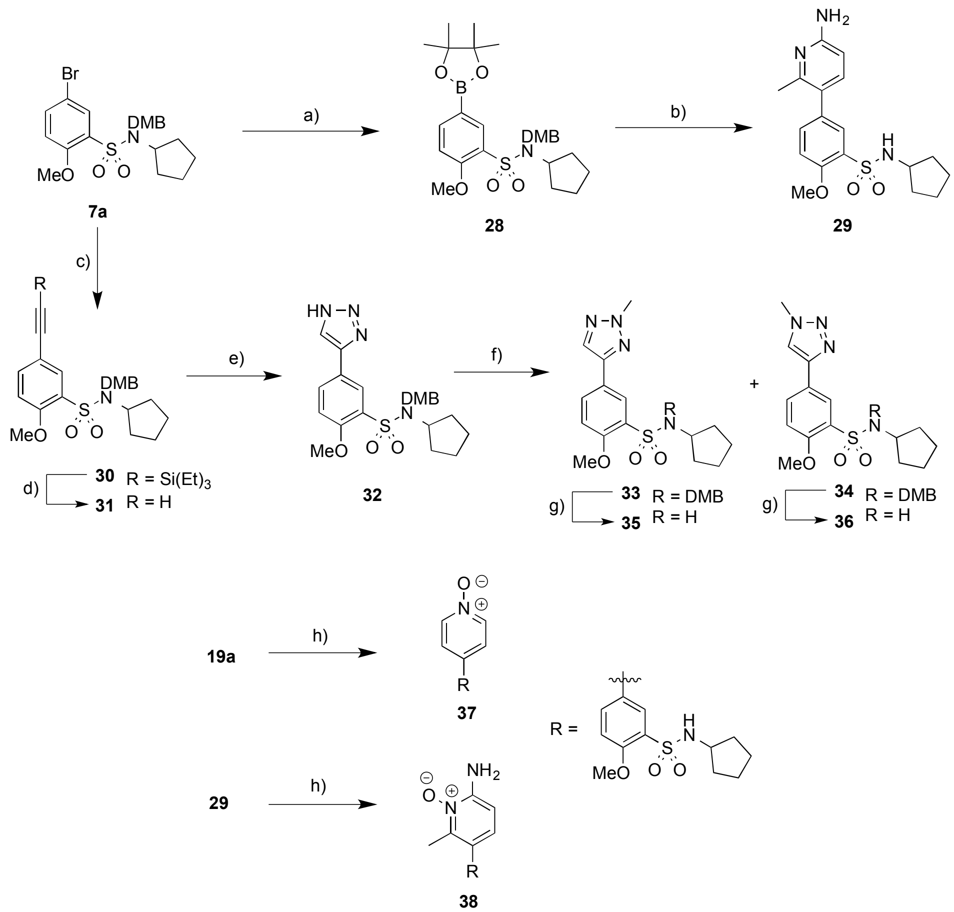
It was envisaged that diverse aryl and heteroaryl functional groups could be incorporated onto aryl bromides **7a** and **7b** by employing a Suzuki-Miyaura cross-coupling reaction. After considerable optimisation, a general method for enabling the Suzuki-Miyaura cross-coupling between **7a** and **7b** and a range of aryl boronic acids and esters, in a parallel fashion, was established using the PEPPSI-SiPr™ pre-catalyst[30] with a dioxane:H_2O:DMF solvent system, in the presence of Cs_2CO_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).[31]



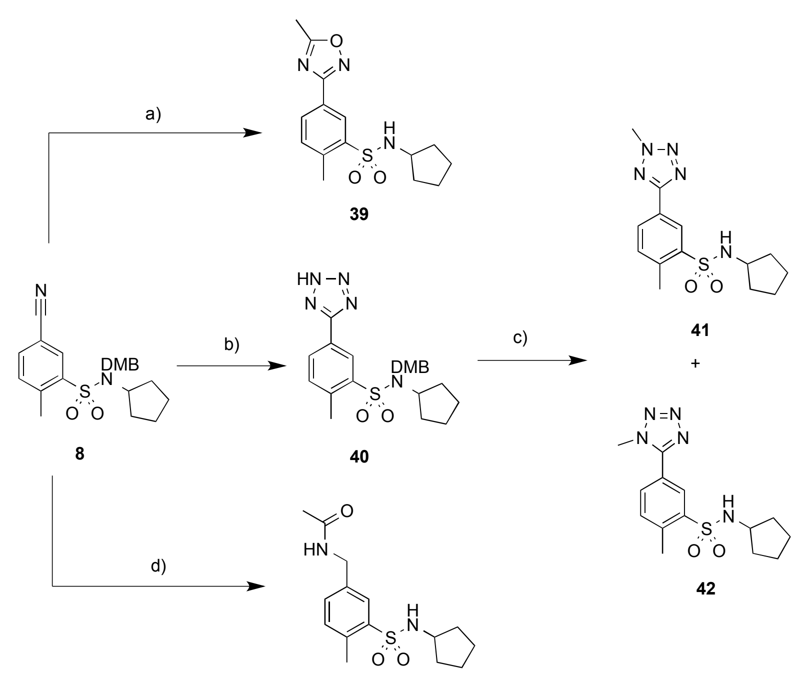
Scheme 2. *Reagents and conditions:* a) i) PEPPSI-SIPr™, Cs_2CO_3, dioxane, H_2O, DMF, 90 °C, microwave, 12 h. ii) TFA, CH_2Cl_2, rt, 2 h.

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 trimethylsilylazide 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 *m*-CPBA providing **37** and **38** respectively (Scheme 3).



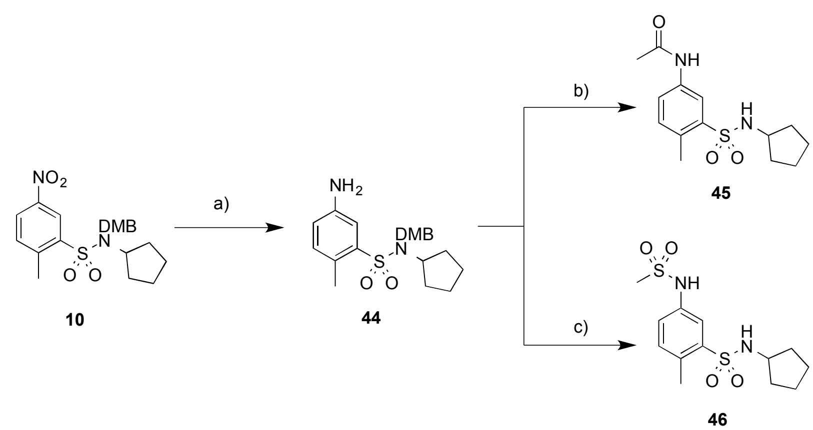
Scheme 3. *Reagents and conditions:* a) B_2pin_2, $Pd(dppf)Cl_2$, PPh_3, KOAc, DMSO, 100 °C, 12 h. b) i) 2-amino-5-bromo-6-methylpyridine, PEPPSI-SIPr™, Cs_2CO_3, dioxane, H_2O, DMF, 90 °C, microwave, 12 h. ii) TFA, CH_2Cl_2, rt, 2 h. c) Triethylsilylacetylene, $Pd(PPh_3)_4$, CuI, Et_3N, THF, 80 °C, 12 h. d) TBAF, THF, 2 h, 0 °C. e) Trimethylsilylazide, CuI, DMF, MeOH, 90 °C, 12 h. f) MeI, $NaHCO_3$, DMSO, rt, 12 h. g) TFA, CH_2Cl_2, rt, 2 h, h) *m*-CPBA, CH_2Cl_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).



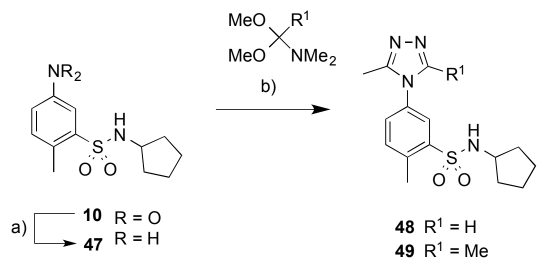
Scheme 4. *Reagents and conditions:* a) i) dimethylacetamide-dimethylacetal, $NH_2OH.HCl$. ii) HCl, dioxane, 2 h, rt. b) i) MeI, $NaHCO_3$, DMSO, rt, 12 h. ii) HCl, dioxane, rt, 12 h. c) NaN_3, DMF. d) i) H_2, Pd/C, MeOH, 'H-cube^{TM}' flow reactor. ii) Ac_2O, EtOAc, HCl. iii) HCl, dioxane, rt, 2 h.

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) H_2, Pd/C, 40 °C, 'H-cube^{TM}' flow reactor. b) i) Ac_2O, EtOAc, HCl, rt, 2 h. ii) TFA, CH_2Cl_2, rt, 2 h c)i) methanesulfonyl chloride, pyridine, CH_2Cl_2, 0 °C, 2h. ii) TFA, CH_2Cl_2, 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).



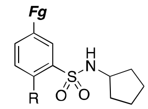
Scheme 6. *Reagents and conditions:* a) H_2, Pt/C, 40 °C, 'H-cube™' flow reactor. b) $AcNHNH_2$, MeCN, AcOH, 50-120 °C, 24 h.

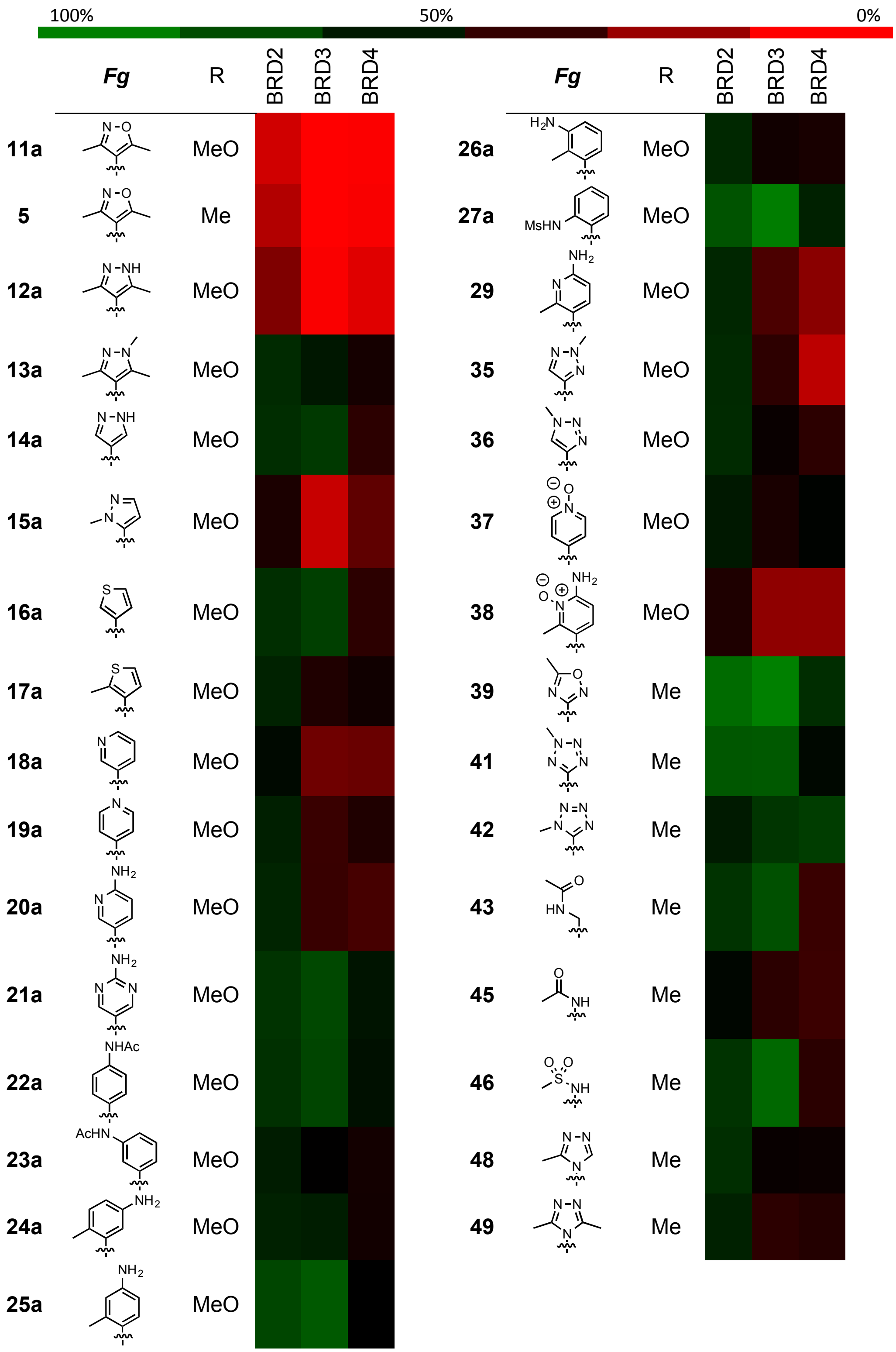
2.2 Biochemical Assays

An ALPHAscreen™ 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 mimetic, 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[TM] binding data expressed as % of control (DMSO) at 5 µM compound concentration.





To confirm and quantify the single point ALPHAscreen™ assay data, the IC_{50}s against BRD3 and BRD4 of 7 selected compounds were determined. Consistent with the single point data and with previously reported data[20] the isoxazole **11a** displayed IC_{50}s of 0.20 μM and 1.2 μM against BRD3 and BRD4 respectively and **5** had IC_{50}s of 0.25 μM and 0.67 μM. Dimethylpyrazole **12a** showed slight selectivity for BRD3 (IC_{50} = 1.32 μM) over BRD4 (2.89 μM) while monomethylpyrazole **15a** showed some selectivity for BRD3 (IC_{50} = 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 (IC_{50} = 4 μM) over BRD3 (IC_{50} = 10 μM). Finally, the IC_{50} data also confirmed the surprising lack of binding exhibited by 1,2,4-triazoles **48** and **49** (IC_{50}s 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 (pK_{BHX}) 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.

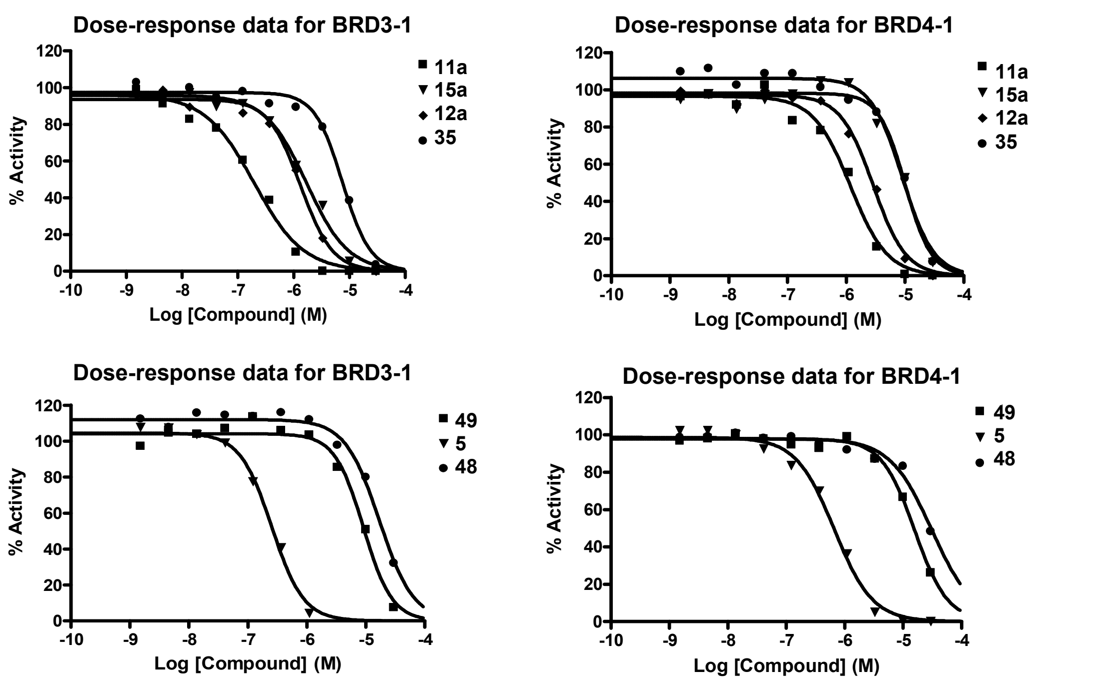


Figure 3. Selected ALPHAscreen™ binding data IC_{50} for assays on BRD4(D1) and BRD3(D1).

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* studies[8,17,34] 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.

Compound	Microsomal Species	Degradation half-life (min)	*In vitro* CL_{int} (µL/min/mg protein)	Microsome-predicted E_H
5	Human	7	246	0.91
	Rat	2*	711*	0.95*
	Mouse	4*	461*	0.91*
49	Human	173	10	0.29
	Rat	107	16	0.29
	Mouse	85	20	0.30
IBET-151 (**2**)	Human	234	7	0.23
	Rat	207	8	0.18
	Mouse	> 247	< 7	< 0.13
(+)-JQ1 (**1**)	Human	13	136	0.84
	Rat	13	129	0.77
	Mouse	7	166	0.78

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 mimetic 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™ 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.

Acknowledgements
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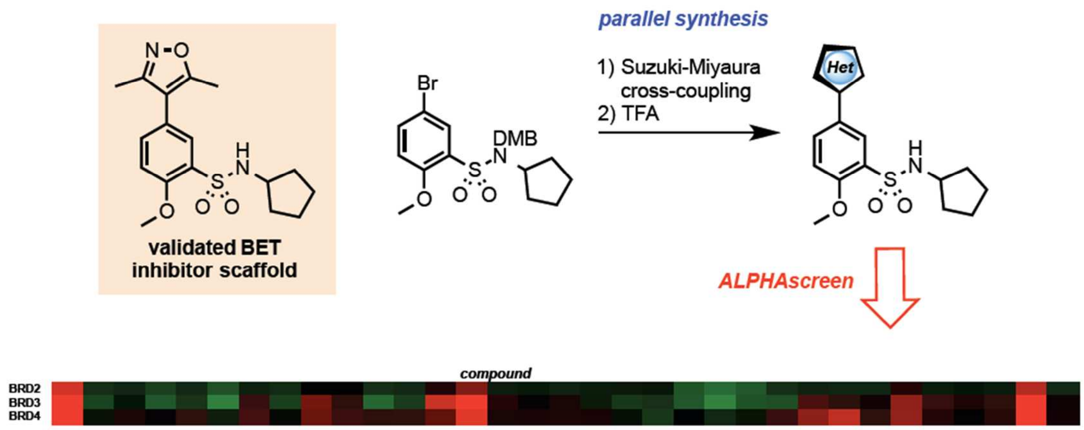

This work provides new insights into a range of acetyl-lysine mimetics as BET bromodomain inhibitors.