Use of N,N-dimethyl ethylamine groups to target D144 of BRD4

UROP research report

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Abstract: Though bromodomain and extraterminal (BET) family proteins are important epigenetic regulating proteins, high sequence similarity makes selective binding challenging. Molecules selective to the N-terminal bromodomain of the human bromodomain-containing protein, BRD4, have only been characterized recently and have spurred on therapeutic applications of BET inhibition. Here, the affinity of a BRD4-selective 1,4,5-trisubstituted imidazole scaffold is improved ~14-fold with the addition of a N,N-dimethyl ethylamine moiety as measured by a fluorescence polarization assay. This improvement is hypothesized to be due to electrostatic interactions with a nearby aspartic acid residue. Limitations of this characterization and conclusion are discussed.

Introduction: Within multi-cellular organisms, there are a myriad of cell-types, each with a specific role. A liver cell and a red blood cell have different phenotypes, but they both contain an identical genome. The epigenome is an overarching system which determines which gene is expressed and to what extent. It regulates expression to allow a cell to choose to differentiate into a red blood cell to transport oxygen or into a liver cell to filter blood.

Genetic information is stored in deoxyribonucleic acid (DNA). These double helix strands of DNA wind around histone octamers, forming chromatin[1]. The tails of these histone proteins extend past the DNA and can bear post translational modifications (PTMs)[2]. One such PTM is the acetylation of histone lysine residues, a part of the epigenetic code[2,3]. To recognize these modifications, epigenetic proteins contain bromodomains[4].

Bromodomains are ~110 amino acid domains that exist on 46 distinct human proteins[5,6]. Of the 61 human bromodomains, the bromodomain and extraterminal domain (BET) family is one well-studied family[5]. This family is comprised of bromodomain-containing-protein 2, 3, 4, and T (BRD2, BRD3, BRD4, and BRDT)[5]. Each member contains two highly conserved bromodomains on the N-terminus and a nonconserved C-terminal domain[7]. A small molecule, (+)-JQ1, was developed in 2010 that inhibits all the bromodomains of the BET family with comparable affinity[5]. With the development of this BRD4 inhibitor, BET inhibition has become an important target for therapeutics against inflammatory and other diseases[8].

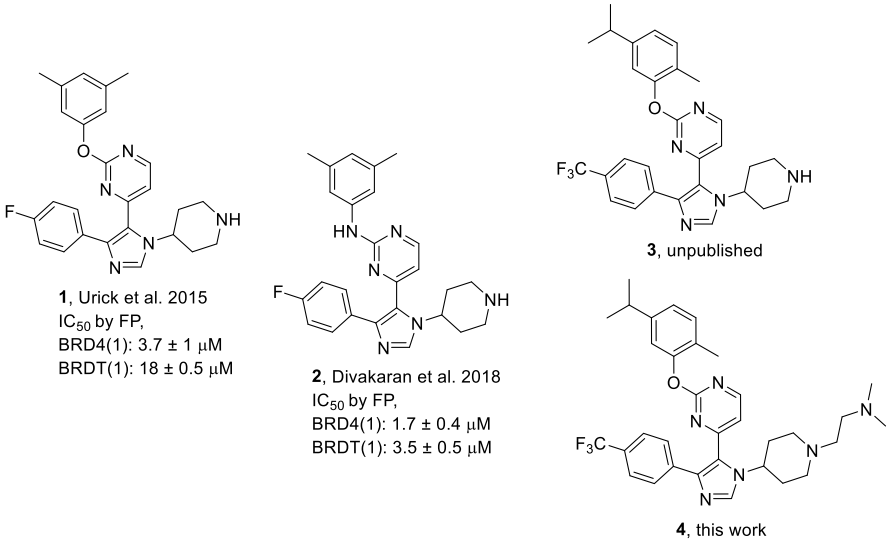

Figure 1 | Previous and lead BRD4(1) selective inhibitors. **3** was synthesized in yet unpublished work by Huarui Cui

Selectivity within the BET family has been challenging, because these bromodomains of the BET family are highly conserved [6]. In 2015, Urick et al. found **1**, a 1,4,5-trisubstituted imidazole, to bind to BRD4 but not BPTF, a bromodomain-containing-protein outside the BET family, see figure (1)[9]. A screen of the interfamily selectivity was done by Divakaran et al. in 2018, in which **1** was found to be selective to the first domain of BRD4, denoted BRD4(1)[10]. Affinity to BRD4(1) was gained by modifying the scaffold to form **2** in the same work. In yet unpublished work, Huarui Cui made improvements to the 1,4,5-trisubsitituted imidazole scaffold, making **3** the current lead molecule.

Results: With acceptable yields in line with the literature, **4** was synthesized. The dehydration reaction to form **7** had lower than normal yields. The whole synthetic pathway is given in figure (2) with yields. ^{1}H NMR confirmed the identity of all intermediates. The ^{1}H NMR of the purified **4**, figure (S1), confirms the product, with some unresolved peaks.

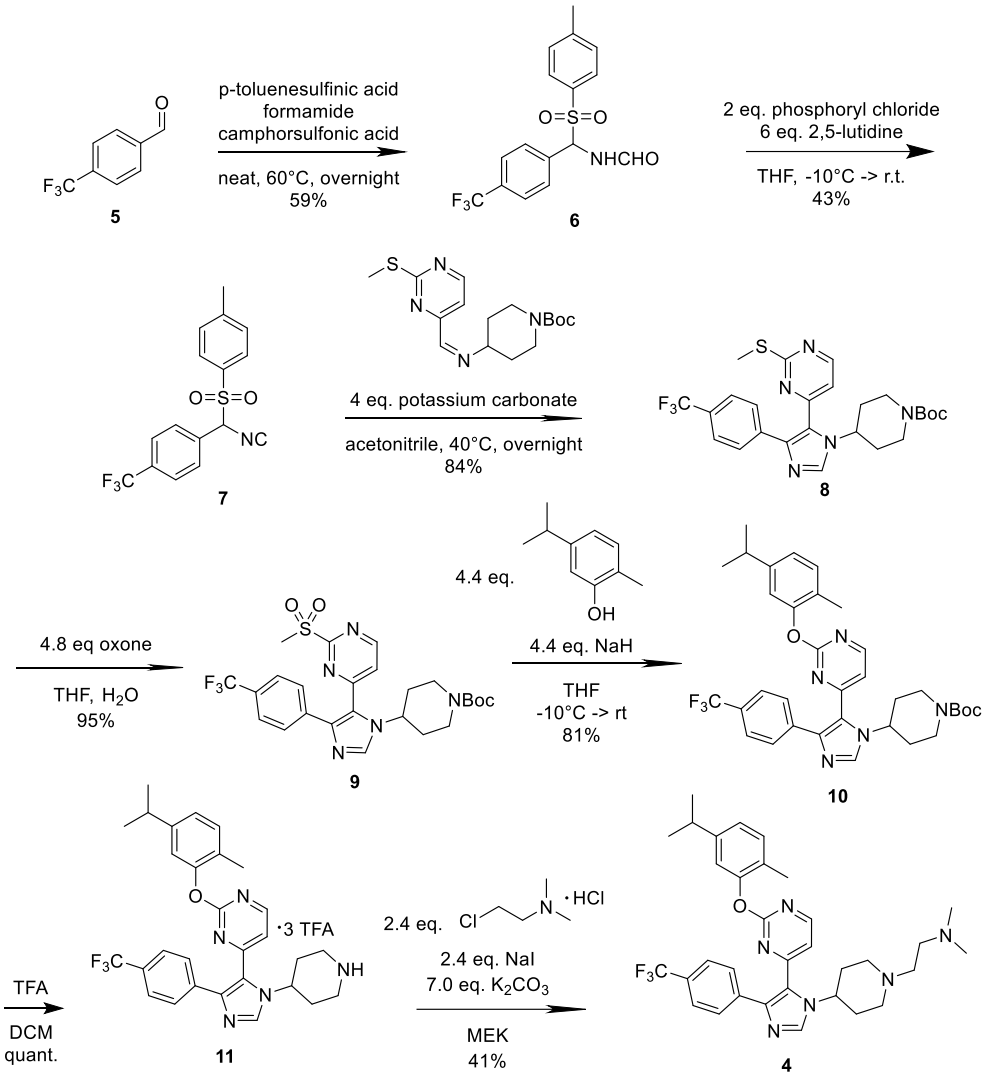

Figure 2 | Synthetic pathway to form **4** with yields achieved

The binding affinity of the molecules was quantified by fluorescence polarization (FP). Because this technique is sensitive to variations in ambient conditions, only molecules on the same plate are

compared. For comparisons to the previous lead molecule and analogs, Huarui Cui kindly supplied previously synthesized molecules, shown in figure (3).

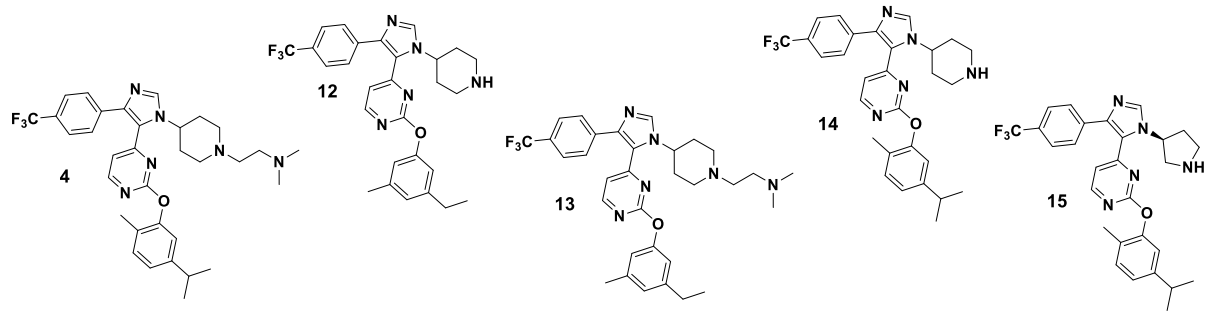

Figure 3 | Molecules tested by fluorescence polarization. **12**-**15** were kindly provided by Huarui Cui

To analyze the effect of **4** containing the N,N-dimethyl ethylamine group, **4** and **12** were tested in a competitive binding FP assay as shown in figure (4A). The binding isotherm is complete for all molecules tested; there are small and consistent error bars that generally increase at lower concentrations; the hill-slopes are shallow, especially compared to (+)-JQ1. **15** was analyzed on the same plate, to investigate the effect of ring size. The affinities from all competition experiments are given in table (1).

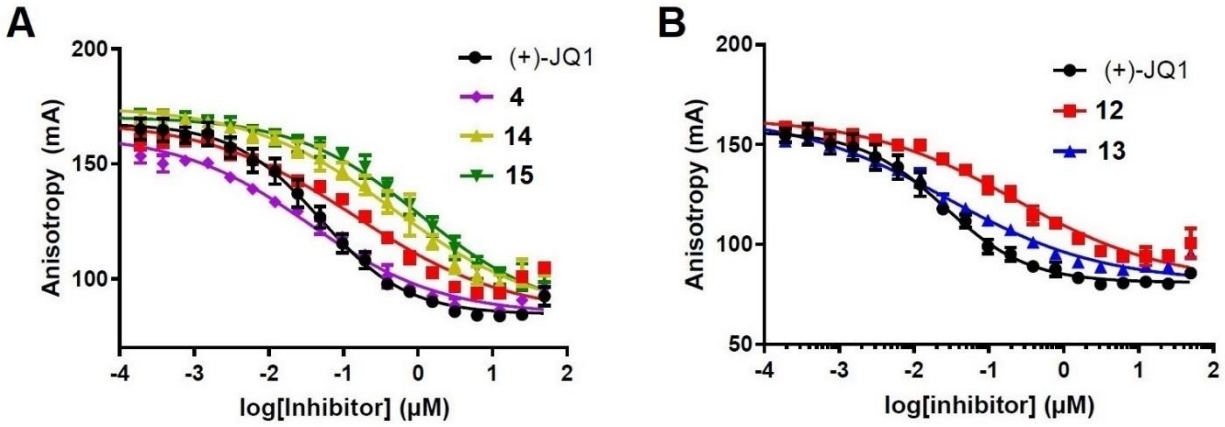

Figure 4 | Competitive binding fluorescence polarization assays of (A) **4**, **15**, and **16** (B) **13** and **18**. Both are against BRD4(1) at 0.23 μM with (+)-JQ1 as a control.

To support the results of figure (4A) with a different phenolic substituent, **12** and **13** were tested under the same conditions. The fitted isotherms are shown in figure (4B). The hill-slopes of the isotherms are shallow, possibly indicating problems in the setup.

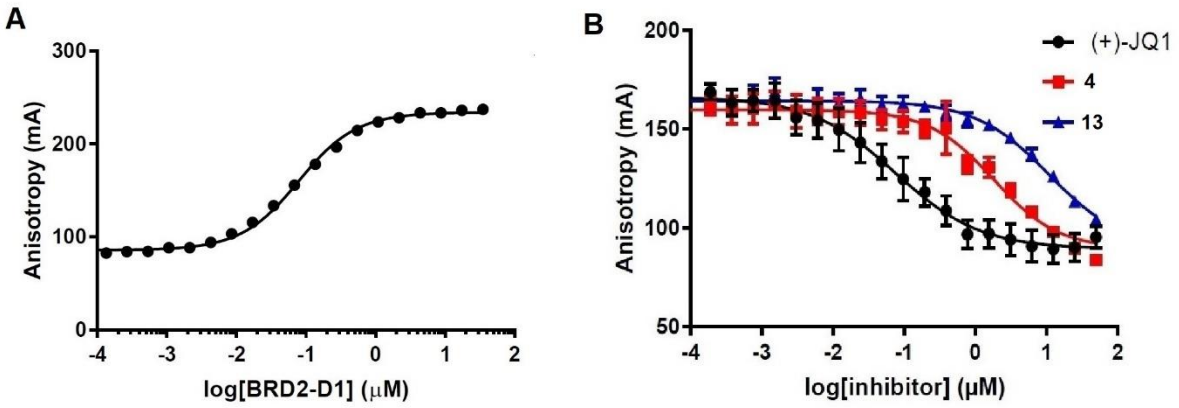

Figure 5 | Fluorescence polarization traces for BRD2(1) binding. (A) Direct binding experiment between the fluorophore tracer and BRD2(1). (B) Competitive binding assay against BRD2(1) of **4**, and **6** with (+)-JQ1 as a control.

To prepare for a competitive binding assay to another BET family member, a direct binding assay was done between the fluorophore and BRD2(1). The isotherm is shown in figure (5A). For 0.015 µM of the fluorophore tracer, 80% of the tracer is bound at a protein concentration 0.273 µM. Thus, this concentration was used for the subsequent competitive binding assay to BRD2(1). To quantify the selectivity of **4** and **13** for BRD4(1) against BRD2(1), the affinity to BRD2(1) was found. The fitted isotherm is shown in figure (5B). The steeper hill-slopes possibly indicate a better setup. Finally, table 1 presents the fitted IC_{50} values from these experiments.

Table 1 | Characteristics of inhibition found by fluorescence polarization. All values are fitted to an experiment with technical triplicates.

To BRD4(1)	(+)-JQ1	**4**	**12**	**13**	**14**	**15**
IC_{50} (µM)	0.049	0.035			0.49	1.1
IC_{50} (µM)	0.026		0.23	0.028		
To BRD2(1)						
IC_{50} (µM)	0.076	1.8		11		

Discussion: There is a ~14-fold increase in affinity to BRD4(1) between **4** and **14**, but a ~8-fold increase between **13** and **12.** Previous crystal structures (PBD ID: 6MH1) have revealed the solvent exposed aspartic acid (Asp144, see figure (6)) near the binding site. Thus, a positively charge moiety such as this amine might make an electrostatic interaction. With these crystal structures, an ethyl linker should put the amine in close proximity to the negative acid residue.

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BRD2(1) 147 ... TMFTNCYIYNKPTDDIVLMAQ ...
BRD3(1) 107 ... TMFTNCYIYNKPTDDIVLMAQ ...
BRD4(1) 131 ... TMFTNCYIYNKPGDDIVLMAE ...
BRDT(1) 100 ... TMFSNCYLYNKPGDDIVLMAQ ...

BRD2(2) 420 ... LMFSNCYKYNPPDHDVVAMAR ...
BRD3(2) 382 ... LMFSNCYKYNPPDHEVVAMAR ...
BRD4(2) 424 ... LMFSNCYKYNPPDHEVVAMAR ...
BRDT(2) 343 ... LMFMNCYKYNPPDHEVVTMAR ...
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Figure 6 | Protein sequences of BET family bromodomains showing homology[11]. D144 of BRD4 and the corresponding residues of the other domains are highlighted

Comparing the IC_{50} values of **4** and **13** between the proteins, the selectivity is ~50 and ~400 respectively for BRD4(1) over BRD2(1). This change in selectivity is due to the phenolic substituent. The bulkier and hydrophobic isopropyl moiety possibly interacts with hydrophobic residues on the surface of the proteins differently.

Because of the sensitivity of FP, there is a dependence on unmeasured ambient conditions. Thus, the experiment must be repeated more than was done here to quantify the binding accurately. However, the general trends and fold-changes should be reliable. Also, the fold-increases on one plate are likely accurate because the experiment occurs at the same conditions with the same technique.

To corroborate the hypothesis that there is an electrostatic interaction between the amine and Asp144, the complex could be crystallized. Solving the x-ray structure would allow the distance between the charges to be found. Using isothermal titration calorimetry, the binding could be more precisely quantified. The affinity of the binding could then be compared to theoretical energetics.

Methods:

Chemical Synthesis: Published gram-scale organic chemistry procedures[9] were used to produce compound **11**.

The Finkelstein reaction to produce compound **4** was as follows: 133 mg (0.154 mmol) of **11** was added to a round bottom flask. 53 mg (0.367 mmol) of 2-chloro-N,N-dimethylethan-1-amine, 55 (0.367 mmol) mg of sodium iodide and 26 mg (1.08 mmol) of potassium carbonate were added, along with a stir bar. 2-butanone was added to form a 0.3 M solution of **11**. The mixture was stirred at room temperature for three days. The reaction was monitored using TLC: an alumina plate developed in 20% methanol in ethyl acetate. The reaction mixture was diluted with water and extracted with ethyl acetate 3 x. The organic phase was dried with magnesium sulfate, filtered and concentrated under vacuum. The resulting solid was purified by reverse-phase HPLC and isolated as a solid

The product of each reaction was confirmed by ^{1}H NMR using deuterated dichloromethane or methanol, depending on the solubility. The synthetic pathway is shown in figure (2), with yields given.

Product Confirmation: 25-30 mg of dried solid product was dissolved in 0.5 mL of either deuterated dichloromethane or deuterated methanol, depending on solubility. ^{1}H NMR spectra were collected on a 500 MHz spectrometer.

Fluorescence Polarization: As described previously[12], the solutions were prepared and transferred to a plate. The data was fit to the Cheng-Prusoff variant given elsewhere[12] using Prism.

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Appendix:

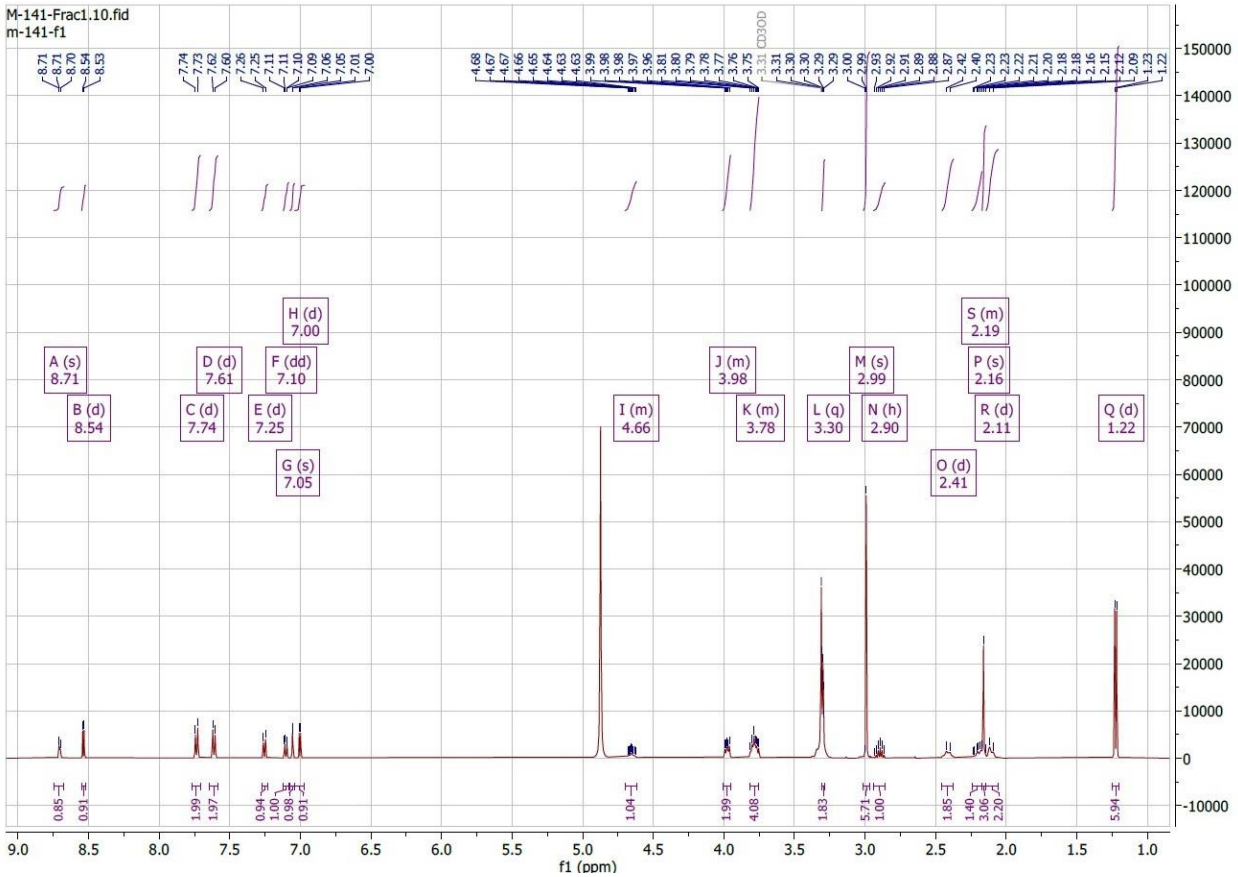
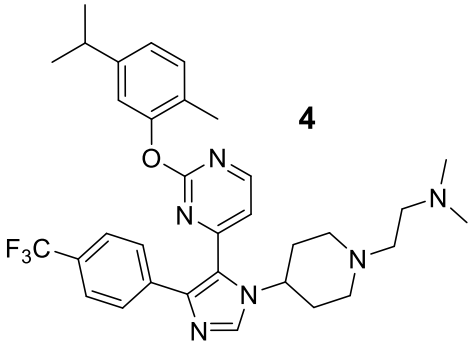


Figure S1 | ^{1}H NMR spectrum of **4** taken on a 500 MHz spectrometer in deuterated methanol



^{1}H NMR (500 MHz, MeOD) δ 8.71 (s, 1H), 8.54 (d, *J* = 5.1 Hz, 1H), 7.74 (d, *J* = 8.2 Hz, 2H), 7.61 (d, *J* = 8.2 Hz, 2H), 7.25 (d, *J* = 7.9 Hz, 1H), 7.10 (dd, *J* = 7.9, 1.8 Hz, 1H), 7.05 (s, 1H), 7.00 (d, *J* = 5.0 Hz, 1H), 4.70 – 4.62 (m, 1H), 4.01 – 3.95 (m, 2H), 3.81 – 3.75 (m, 4H), 3.30 (q, *J* = 1.6 Hz, 2H), 2.99 (s, 6H), 2.90 (h, *J* = 7.0 Hz, 1H), 2.41 (d, *J* = 12.0 Hz, 2H), 2.24 – 2.17 (m, 1H), 2.16 (s, 3H), 2.11 (d, *J* = 12.8 Hz, 2H), 1.22 (d, *J* = 6.9 Hz, 6H).