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ARTICLE TYPE

Direct photocapture of bromodomains using tropolone chemical probes

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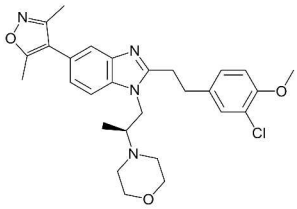
Medicinal chemistry techniques, including structure-based molecular design, fragment replacement and synthetic library enablement, were used to create potent inhibitors of bromodomain and extraterminal domain (BET) and CREB binding protein bromodomains. One inhibitor featured the homoaromatic tropolone methyl ether motif as a mimic of acetyl lysine, as confirmed by X-ray crystallography. The intrinsic photoreactivity of the tropone fragment was harnessed successfully to directly photolabel recombinant bromodomains which inspired further development of a clickable probe to assess BRD4 target engagement by (+)-JQ1 as a representative BET inhibitor in a complex proteome. The antimitotic natural product colchicine and related natural products that contain the tropolone ring system were also found to be bromodomain inhibitors, and surprisingly, our probe was shown to photolabel tubulin. These results highlight the caution that should be exercised when considering the selectivity of pharmacological agents, and photoreactive chemical probes should be assessed for their intrinsic ability to directly label their biological targets with a view to creating useful chemoproteomic tools.

There is intense interest in the biology of 'reader' bromodomain proteins of the histone code. Bromodomain-containing proteins recognise acetyl lysine marks associated with epigenetic information transfer, and small molecule inhibitors that perturb these interactions are used to decipher their associated biology.[1] One such example, that of bromodomain and extraterminal domain (BET) inhibitors, has illustrated the therapeutic potential of this class, particularly in cancer.[2, 3] An additional role for the bromodomain BRD4 was discovered recently that involved modulation of the signalling response to DNA damage *via* chromatin insulation.[4]

We recently described a framework to support unbiased target validation, which included the concept of the '4 pillars' when using chemical probes: sufficient exposure at the site of action (Pillar 1); proof of target engagement (Pillar 2); expression of functional pharmacology (Pillar 3); and proof of perturbation of a relevant phenotype (Pillar 4).[5] Indeed, the BET case history, including the discovery of inhibitors (+)-JQ1,[6] I-BET[7] and PFI-1,[8] nicely illustrated the importance of achieving all 4 pillars to unequivocally link target modulation to therapeutic effects.[5]

In addition to our interests in BET proteins, we are also exploring the pharmacology of the related bromodomain-containing protein CREB (cyclic-AMP response element binding protein) binding-protein (CREBBP). There is a need to develop selective CREBBP inhibitor probes to further elucidate the complex biology of this protein. In the course of our medicinal chemistry programme we uncovered a novel tropolone methyl ether mimetic of acetyl lysine which directly photolabelled both bromodomain-containing protein 4 (BRD4, domain 1) and CREBBP recombinant bromodomains. This enabled further development of an alkyne-tethered clickable probe to assess bromodomain engagement by small molecule inhibitors in a cancer cell line.

A previously disclosed isoxazole CREBBP inhibitor SGC-CBP30 (developed by the Structural Genomics Consortium) served as the starting point in our design of novel probes (Fig. 1).[9, 10] The dimethyl-isoxazole head group is a common acetyl lysine mimic used in various bromodomain inhibitors and our desire was to explore replacements of this fragment.[11-16]



SGC-CBP30
CREBBP IC_{50} 69 nM
BRD4 40-fold selective by ITC

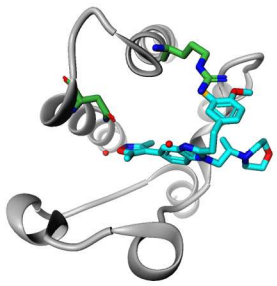


Fig. 1 CREBBP inhibitor SGC-CBP30 from the Structural Genomics Consortium and X-ray crystal structure with CREBBP (PDB code 4NR7).[9, 10]

Disconnection across the isoxazole-benzimidazole bond of the simpler CREBBP inhibitor **1**[10] suggested that Suzuki library chemistry would be an efficient means to explore alternative head groups (Fig. 2). Aromatic bromide monomers were chosen based on bioisosteric replacement using BROOD (OpenEye Scientific Software Inc., Santa Fe, NM) that takes into account the shape, fundamental chemical connectivity and electrostatic similarity to the initial structure, and NEAT (Novel and Electronically-

equivalent Aromatic Template) that uses pre-calculated quantum mechanical charges to search for aromatic rings with similar electrostatic potentials, dipoles and hydrogen bonding capabilities to the query template (dimethyl-isoxazole in this case).[17] A library of 137 compounds was created using Suzuki coupling chemistry on the simpler boronic ester template **2** following the BROOD-NEAT protocol, and screened against CREBBP (FRET assay – see ESI†). This yielded a pyridone hit (a known acetyl lysine mimic, see ESI†)[18] and an unexpected homoaromatic tropolone methyl ether PF-06670015 (**3**, Fig. 2, CREBBP IC_{50} 208 nM). Isothermal titration calorimetry (ITC) confirmed CREBBP binding of **3** (K_d 501 nM; stoichiometry 1:1 - see ESI†).

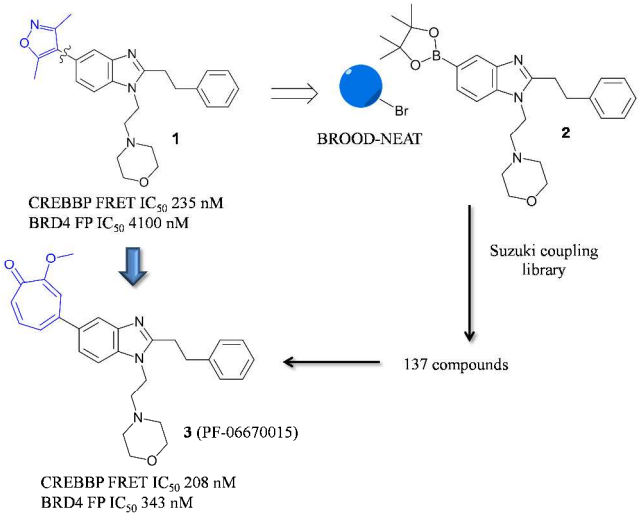


Fig. 2 Library design of bromodomain inhibitors and discovery of the tropolone methyl ether **3**.

A crystal structure of **3** and CREBBP (Fig. 3a) validated the expected binding mode of the inhibitor (similar to the deposited structure of SGC-CBP30 in the PDB, code 4NR7, Fig. 1), which confirmed the tropolone methyl ether motif as an acetyl lysine mimic. The tropolone carbonyl oxygen makes a hydrogen bond to the key asparagine residue (Asn1168), a conserved interaction seen for other bromodomain inhibitors. The carbonyl oxygen atom and that of the methyl ether make hydrogen bonds to a stable water molecule (Water A). The tropolone and benzimidazole planes are oriented at a 50° angle allowing the benzimidazole nitrogen at the 3-position to make optimal hydrogen bonding interactions with Arg1173, causing the phenethyl substituent to orient along the hydrophobic northern channel. Arg 1173 adopts an edge-to-face orientation with the ligand benzene ring, as opposed to the more traditional cation-π interaction seen for SGC-CBP30 and another recently published inhibitor from the SGC.[19] Additionally, there are weak σ-π interactions, one between a tropolone -$OC\underline{H}_3$ hydrogen atom and Phe1111, and the other between Pro1110 Cα H and the benzene ring of the benzimidazole.

3 was also shown to be an inhibitor of BRD4 using a fluorescence polarization (FP) assay (IC_{50} 343 nM, using a fluorescently tagged derivative of PFI-1) and ITC (K_d 110 nM). BROMO*scan*® profiling (which uses affinity isolation of DNA-tagged bromodomains – see ESI†) showed **3** to be a submicromolar inhibitor of additional bromodomains: BRD2, BRD3, BRDT and EP300.

3 was crystallized with BRD4 to investigate the binding mode. As observed in the CREBBP crystal structure, the tropolone makes the conserved hydrogen bond interactions with the BRD4 Asn140 residue. However, the rest of the binding interactions are different. The binding pocket water molecule (Water E) makes a critical bridging interaction between the carbonyl oxygen atom of Pro82 and the benzimidazole 3-position nitrogen atom. This key interaction, along with the hydrophobic shielding provided by the Trp81 residue, stabilize and orient the phenylethyl substituent now to the southern pocket, and places the morpholine motif in the solvent-exposed region.

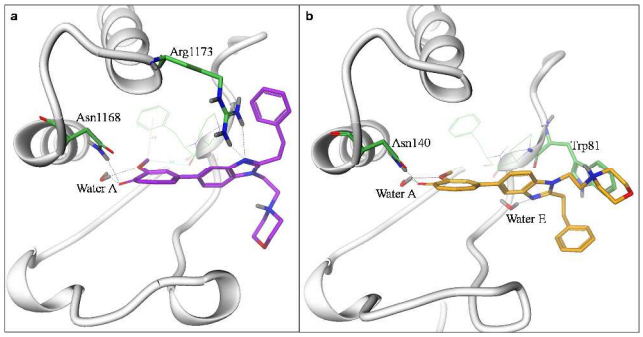


Fig. 3 X-ray crystal structures of **3** (PF-06670015) with a) CREBBP (PDB code 4WHU) and b) BRD4 (PDB code 4WHW). Key residues and the conserved water molecules are highlighted.

Re-introducing the *para*-methoxy group (present in SGC-CBP30) to provide **4** had little effect on BRD4 and CREBBP potency, while the regioisomeric derivative **5** (originally prepared in the Suzuki library) was significantly less potent (Fig. 4) as may be expected from the crystal structures in Fig. 3.

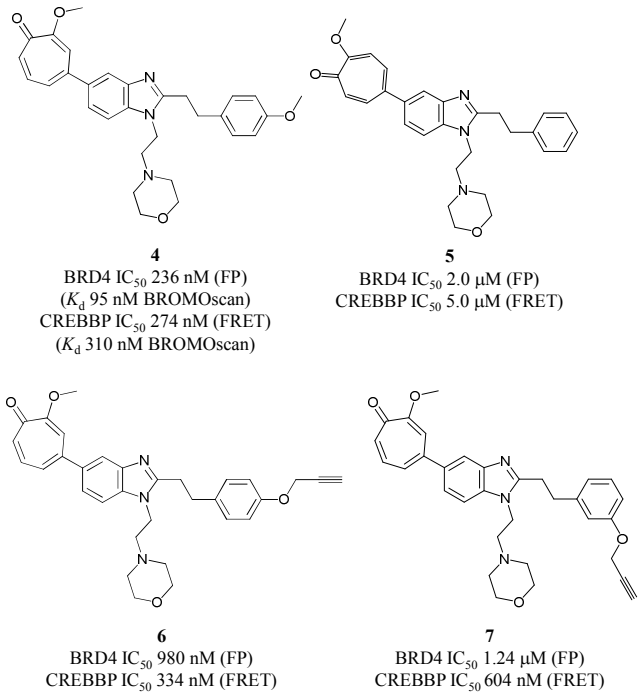


Fig. 4 Structures and bromodomain potency of tropolone methyl ether inhibitors

Colchicine (Fig. 5) is an antimitotic natural product that contains

the tropolone methyl ether fragment and we therefore surmised it may be an inhibitor of bromodomain-containing proteins. BROMO*scan*® profiling of colchicine showed it to be a weak inhibitor of BRD4 (confirmed in the BRD4 FP assay with IC_{50} 52 μM – see also ESI† for full BROMOscan data). Remarkably, a recent publication also confirmed colchicine as a BRD4 inhibitor.[20] In this work, a virtual screen of 7 million molecules against the BRD4 crystal structure yielded a number of hits including colchiceine (des-methyl colchicine) and colchicine itself (K_d 46 μM and 20 μM respectively – see Fig. 5). Colchicine was crystallized with BRD4 (PDB code 4LZR)[20] and the tropolone methyl ether motif overlays closely with that in the structure of **3** (Fig. 5). These results suggest that caution should be exercised when interpreting data from experiments using colchicine at these, or higher, concentrations due to the potential for bromodomain biology modulation. We also showed that another natural product, trimethylcolchicinic acid was an even more potent inhibitor of BRD4 (Fig. 5). This implies that colchicine natural product chemistry and the use of synthetic biology techniques could be used to produce pharmacological modulators of the bromodomain family.

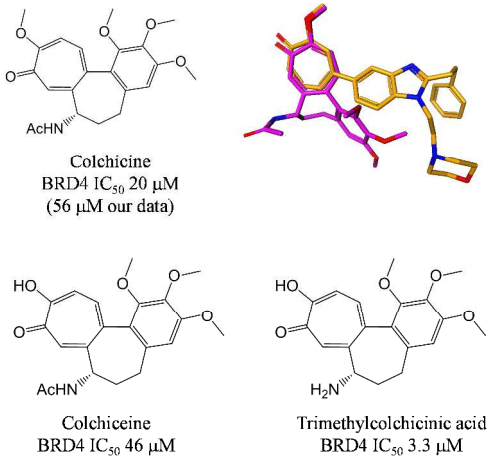


Fig. 5 Structures and BRD4 inhibition of colchicine, colchiceine and trimethylcolchicinic acid. Overlay of **3** (gold, PDB code 4WHW) and colchicine (magenta, PDB code 4LZR) in BRD4 (protein removed for clarity).

Colchicine is photosensitive, and has previously been harnessed to effect direct photolabelling of its target protein tubulin in cells.[21, 22] Inspired by this observation, we hypothesised that tropolone methyl ether **4** could be a viable photoaffinity label for bromodomains. Treating BRD4 or CREBBP with **4** (499 Da) and 365 nm irradiation yielded a +485 Da adduct to each protein, presumably formed by addition of the probe to the protein with concomitant demethylation (Fig. 6). An additional very minor peak corresponding approximately to an adduct of the full mass of the probe was also present in each case, but it could not be excluded that these were artefacts of electrospray. The mechanism of photolabelling, and the residues labelled in the bromodomain binding sites are unknown at this point, and the low efficiency of labelling may complicate site recognition; however, this is not essential to the application described below.

As a step toward defining potential mechanisms of photoreactivity, probe **4** was similarly irradiated in aqueous Tris buffer. Separate adducts of **4** to water and Tris were detected, each formed with a concomitant loss of 14 Da (see Fig. S2 in ESI†). As this presumptive demethylation also accompanied protein photolabelling, the chemistry underlying the reactions of **4** with water and Tris must resemble that occurring between **4** and the bromodomains. These observations, which are receiving further investigation in our group, suggest a two-step labelling process. Light-mediated photoactivation may create a reactive intermediate that subsequently labels the protein (for example, intramolecular [2+2] cycloaddition chemistry of tropolone derivatives is known to create strained and reactive 5,4-fused ring systems).[23, 24]

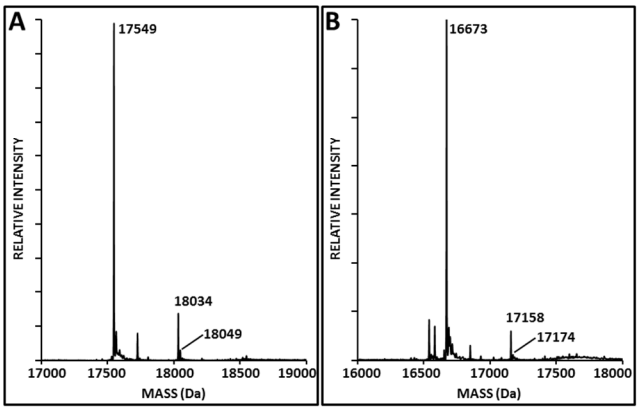


Fig. 6 LC-MS showing protein mass spectra for (A) recombinant BRD4 and (B) recombinant CREBBP following irradiation at 365 nm in the presence of probe **4**. Probe **4** has a molecular mass of 499 Da, and the principal adduct peak detected in each protein spectrum corresponds to a mass increase of 485 Da.

As mentioned above, clickable probes have found significant utility in target validation to confirm target engagement in physiologically relevant contexts. To this aim, an alkyne click handle was appended to the *para*- and *meta*-positions of the benzene ring as they appeared to be solvent exposed (and adding the *para*-methoxy group had little effect on the potency of **4**), to enable copper-mediated azide-alkyne cycloaddition (CuAAC)[25, 26] with an azide-containing reporter tag. Clickable tropolone photoprobes **6** and **7** retained inhibitory activity against CREBBP and BRD4 (Fig. 4).

As a proof-of-principle experiment we were able to photolabel BRD4 spiked into K562 cell lysate using **7** (**6** did not label BRD4 as efficiently), followed by a CuAAC reaction with the trifunctional TAMRA-biotin azide, streptavidin enrichment and visualisation by Western blot (Fig. 7). Competition using the BET inhibitor JQ1[6] validated the approach to assess specific bromodomain engagement in a complex proteome. Variability in the amount of competition was seen in the assay, potentially due to the somewhat capricious nature of covalent bond formation during the photoactivation. Another complicating factor is the difficulty of a reversible probe, such as JQ1, to compete the covalent reaction that results when the probe is photoactivated. Unfortunately, we were unable to convincingly detect endogenous BRD4 in live cells, possibly due to low abundance of the protein coupled with the difficulty in identifying a high quality anti-BRD4 antibody (which we believe is a common issue in the field) that resulted in many non-specific bands being identified, thus complicating interpretation.

Since colchicine is known to photolabel tubulin, we hypothesized that our tropolone probes may also photolabel tubulin. Probe **7** was shown to photoaffinity label tubulin in K562 lysate (Fig. 8) and we sought to test if this labelling could be competed with the non-clickable version of **7** (i.e. **4**).

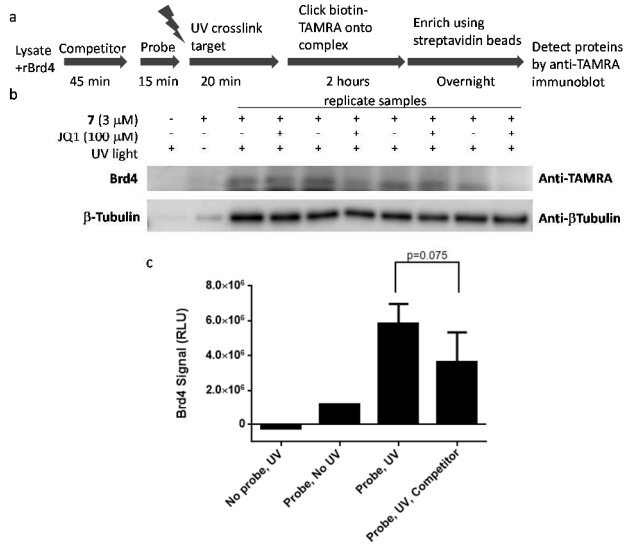


Fig. 7 Labelling and enrichment of recombinant BRD4 from K562 lysate. a) Protocol for labelling, enriching, and detecting protein; b) Immunoblot for proteins enriched by **7** from cell lysate; c) Quantification of recombinant BRD4 labelled by probe (from b). Two-tailed t-test from 4 replicate sets results in p-value of 0.075.

In standard competition studies **4** did not compete with **7**, but we found that photoactivating **4** in the presence of K562 lysate prior to addition of the clickable probe **7** (and further photoactivation) resulted in competition of tubulin enrichment (Fig. 8). This finding confirms the specificity of labelling tubulin with **7** and suggests the likelihood that the reduced competition seen with reversible inhibitors in Figures 7 and 8 is in part due to the one-way direction of the labelling reaction when using a photoreactive probe. The labelling of tubulin by our tropolone probes may also hinder isolation of endogenous bromodomains in live cells, suggesting further probe optimisation is likely required. Further work in our group includes the assessment of drug occupancy of other bromodomains to measure selectivity in physiologically-relevant contexts, especially human primary cells.

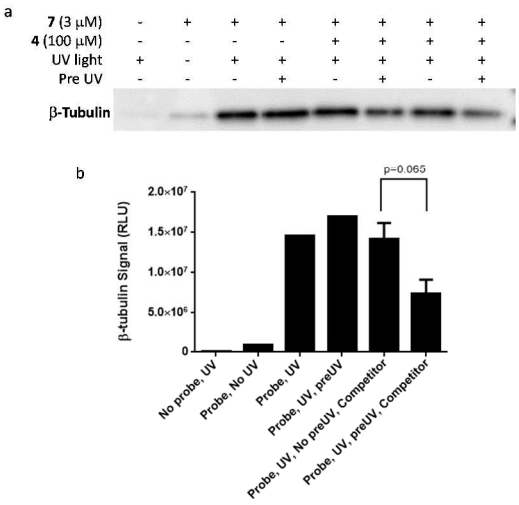


Fig. 8 Labelling and competition of β-tubulin from K562 lysate. a) K562 cell lysate incubated with **4** (non-clickable), exposed to UV, incubated with **7** (clickable), exposed to UV, biotin-TAMRA-azide clicked, streptavidin enriched, β-tubulin detected by immunoblot. Only samples with non-clickable probe exposed to UV light compete for labelling with probe; b) Quantification of signal from immunoblot, two-tailed t-test results in p-value of 0.065

Conclusions

The tropolone methyl ether motif was shown to be an acetyl lysine mimic which photoaffinity labelled bromodomains directly. We have shown how computational techniques such as BROOD and NEAT can enable a bioisosteric switch from dimethyl-isoxazole to a novel tropolone pharmacological replacement. Our work also discovered that colchicine inhibits BRD4, a finding recently confirmed by another group. Somewhat surprisingly, we found that the tropolone natural product trimethylcolchicinic acid is an even more potent inhibitor of BRD4. In some ways, the preponderance of acetyl-lysine replacements suggests that many pharmacological tools, drugs and drug candidates should be assessed for their ability to bind bromodomains.

The design and preparation of a clickable tropolone methyl ether bearing an alkyne tag enabled an assessment of BRD4 engagement by JQ1 in a complex proteome. In general, we believe intrinsically photosensitive ligands should be assessed for their ability to directly photolabel their cognate targets, so enabling target identification, validation, selectivity and interactome analyses. The development of selective inhibitors of CREBBP and the use of these inhibitors in cell-based phenotypic screens will be the subject of a future publication.

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Notes and references

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Tropolone probes directly photoaffinity label bromodomains