Evaluation of linker length effects on a BET bromodomain probe†

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Fueled by the therapeutic potential of the epigenetic machinery, BET bromodomains have seen high interest as drug targets. Herein, we introduce different linkers to a BET bromodomain benzodiazepine ligand (I-BET762) to gauge its implications in the development of hybrid drugs, imaging probes and small molecule drug conjugates. Biophysical studies confirmed minimal disruption to binding of the BRD4 cavity by the synthesized entities, which includes imaging probes. Target engagement was confirmed in a cellular context, but poor membrane diffusion was found despite efficient localization in the nuclei after membrane disruption. Our study highlights challenges and opportunities for the successful design of benzodiazepine-derived drug-delivery systems.

The epigenome is characterized by covalent post-translational modifications (PTMs) of histone proteins and DNA that indirectly affect transcription, DNA repair and replication.1,2 Histone PTMs are dynamically controlled by chromatin-modifying enzymes in a highly-regulated manner. These regulations involve enzymes that apply (writers) or remove (erasers) the covalent modifications to specific aminoacids in histone proteins, as well as conserved protein interaction domains that recognize (read) and bind to those chemical modifications, which subsequently translates them in the context of chromatin reorganization and transcriptional control.3 Proteins of the bromo and extra-terminal (BET) family are among the readers of epigenetic PTMs. They recognize acetylated lysine (KAc) residues on histone tails by means of their evolutionary conserved bromodomain (BRD) modules that facilitate aggregation of transcriptional complexes to chromatin.4 Competitive displacement of BET proteins from chromatin with small-molecule inhibitors results in down-regulation of growth-promoting genes, such as the MYC oncogene, which leads to cell cycle and growth arrest, which in turn offers opportunities for therapeutic intervention in cancer.

Potent inhibitors of BET BRD proteins, such as (+)-JQ1 and I-BET762 (compounds 1 and 6 in Fig. 1), have been reported.5,6 The chemical scaffolds of these lead BET inhibitors have since been the subject of optimization with regards to BDR selectivity and activity.7 The excitement emerging from the preclinical success of several BET inhibitors, supported by potent antitumor activities in a wide range of genetic contexts, led to rapid clinical translation of these molecules. However, this translation has been hampered by toxicity, typically related to off-target effects.8 The targeted delivery of BET inhibitors and the development of hybrid constructs may circumvent the shortcomings of BET inhibitors. However, the feasibility of these approaches depends on the design of appropriate linkers and the tolerance of BET BRDs towards derivatized ligands. Here, we report on the design, synthesis and biological profiling of a focused set of I-BET762-derived entities that can be readily functionalized for either imaging and/or therapeutic purposes.

As a first step, we installed different moieties onto the eastern region of 6. X-ray crystallography studies have shown that the amide moiety of 6 sits in a solvent-exposed region in

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**Fig. 1** Structures of BET bromodomain inhibitors.
BET BRDs, which motivated the exploitation of that region for the installation of chemical handles. Chemical syntheses for the required chemical matter were performed through established synthetic procedures (Fig. 2). In short, a propyl (8) or polyethylene glycol (9 and 10) side chains were installed through amide bond formation chemistry prior to tethering of fluorescein to afford compounds 14–16.

First, by focusing exclusively on the influence of the spacer’s addition, we measured its in vitro affinity effect towards BET proteins. By using thermal shift assays we measured temperature shifts ($\Delta T_m$) for 11 against all BRD modules of the human BET family, which were comparable, albeit lower, to those observed for JQ1 and the parent chemotype I-BET762 (Fig. 3a and Table S1, ESI†). Notably no binding outside the BET family was observed. This was further validated in isothermal titration calorimetry experiments against the first and second BRDs of BRD4 [BRD4(1) and BRD4(2), respectively], against which the compound exhibited dissociation constants ($K_D$) values of 53 and 50 nM, respectively (Fig. 3b and Table S2, ESI†), which are comparable to those reported for 6. To further establish that the added linkers would not interfere with target binding, but would allow for the introduction of a fluorescent label, we determined the X-ray structure of BRD4(1) in complex with 11 at high resolution. The compound was well resolved in the density map and engaged the protein in the typical KAc-mode with the triazolo group sitting within the cavity and interacting with Asn140 through a hydrogen bond. The classic network of water molecules within the BRD cavity was retained (Fig. 3c and Table S4, ESI†). Moreover, the protecting tert-butyloxycarbonyl (Boc) group was resolved in the density in two conformations, pointing away from the BRD cavity, and trapped between two molecules in the asymmetric unit (Fig. 3d).

Encouraged by our results, we wondered if our functionalized compounds could directly engage BET proteins in a cellular context. We used fluorescent recovery after photo-bleaching, a well-established technique for assessment of on-target engagement, previously used on BET proteins. By using parental chemotype 6 as positive control we confirmed that a functionalized entity, such as 11, can engage full length BRD4 in cells and displace it from chromatin. Although the parental scaffold readily displayed rapid recovery ($t_{1/2} = 7.4$ seconds) after photo-bleaching, which suggests direct target engagement, 11 did not elicit a similar effect at 1 µM (1 h treatment, Fig. S1, ESI†). However a 5 µM, overnight treatment resulted in increased recovery ($t_{1/2} = 4.9$ and 5.7 seconds for 6 and 11, respectively, Fig. 4).

After having established that the installed linker does not perturb binding to BET BRDs, we explored how our fluorescent analogues behave in vitro (14–16, Fig. 2). We evaluated the

Fig. 2  Synthesis of I-BET762 derivatives equipped with different spacers and a fluorophore. HBTU = 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate. DIPEA = N,N-diisopropylethylamine. THF = tetrahydrofuran. TFA = trifluoroacetic acid. NHS = N-hydroxysuccinimide. DMF = N,N-dimethylformamide.

Fig. 3  In vitro validation of 11 binding. (a) Thermal shift ($\Delta T_m$) data with BET and other BRDs; (b) isothermal titration calorimetry in solution binding with BRD4(1) and BRD4(2); (c) structural engagement of BRD4(1) by 11; (d) packing of 11 between two BRD4(1) molecules.
activity of these chemotypes against BRD2(1), BRD3(1), BRD4(2) and BRD4(1) in AlphaScreen™ assays (Fig. 5 and Table S3, ESI†). All compounds inhibited the target proteins despite the presence of a bulky substituent, such as fluorescein. The inhibitory potency data for BRD4(1) and BRD4(2) proteins were the most similar. PEGylated probes 15 and 16 displayed a marked decrease in potency against BRD4(1) relative to 6 (Fig. 5) with an IC₅₀ value of 5.2 µM (12-fold decrease) and 2.8 µM (7-fold decrease), respectively. This observation may be explained with an entropic penalty that results from the installation of flexible side chains in 6.11 Interestingly, compound 14 that comprises a simple propyl chain as spacer inhibited BRD4(1) with high potency (IC₅₀ = 0.85 µM). Analysis of the inhibitory potency of compounds across the different proteins showed no correlation to spacer composition. While 16 was 16-times more potent (IC₅₀ = 0.24 µM) than 6 (IC₅₀ = 3.9 µM) against BRD2(1), the same compound was unable to induce competitive peptide displacement in BRD3(1) at concentrations as high as 100 µM.

To further validate our fluorescent-based probes in a cellular system, we probed the intracellular localization of the fluorescein-labelled derivatives. LNCaP cells have been shown to be responsive to the inhibition of BET BRDs and particularly to 6.11,12,13 We therefore used LNCaP cells as an in vitro model system for this exploratory assay. In a preliminary flow-cytometry experiment the rate of compound internalization was measured as the total number of cells that incorporated fluorescein. After 2 h treatment with 10 µM of 16, only 0.8% of the total cells were positive for fluorescein. When the dose was increased to 50 µM, 36.6% of the population of cells was fluorescently labelled. However, at these high concentrations treatment with the fluorescein-linker that does not have a BET-targeting ligand (Fig. 6a) also resulted in 12.6% of fluorescein-positive cells (Fig. S2, ESI†). This data suggests that most of the probe internalization at 50 µM was non-specific. Non-functionalized 6 (with no fluorescein) was used for normalization to account for possible effects in cells induced by compound treatment.

As an orthogonal approach, imaging experiments with confocal microscopy confirmed our previous findings. LNCaP cells were incubated in the presence of 10 µM of 16 and 17 for 2 h before images were captured. The amount of internalized dye was minimal in 16-treated cells, yet greater than for control 17 without the BET BRD ligand (Fig. S3, ESI†). It has been reported that fluorescein has a high rate of fluorescence quenching and low photo-stability, which could explain the poor signal observed.14 To investigate this further, the cellular membrane was disrupted with Triton X-100 detergent prior to incubation with conjugates 16 and 17. In these conditions all cells treated with 16 showed a pronounced fluorescent signal, whereas in samples treated with 17 no staining was observed (Fig. S3, ESI†). In addition, the fluorescent signal of 16 was substantially higher in the nuclei in which BET-BRD proteins are localized, which suggests efficient targeting in cells. Our results fully corroborate previous findings where intracellular targets were revealed by employing BRD-directed optical probes,15–17 despite their impaired permeability.

The same trend was observed when incubating cells with the fluorescent conjugate 14, which has only a 3-atom spacer. Fluorescein derivative bearing no BET-targeting ligand (depicted in Fig. 6) showed no internalization nor nuclei co-localization, whereas the fluorescein-labelled 14 showed internalization in live LNCaP cells with an intact cellular membrane, and nuclear co-localization as expected (Fig. 6), although with a modest fluorescent intensity. Single-time point confocal images were taken after 1 and 4 h incubation with 10 µM doses and no differences were observed.

Altogether, the cellular data of 14 and 16 excluded the possibility for fluorescein photo quenching. Moreover, the data demonstrated a lack of correlation between spacer length and the rate of cellular internalization, in agreement with the biochemical assays for inhibitory potency. To understand why fluorescent probes were effective only when cellular membranes
were disrupted, we employed a parallel membrane permeability assay to study compound diffusion across the cell membrane. The Pe values (10^{-6} \text{ cm s}^{-1}) for derivatised compounds 16 (0.22), 15 (0.36), 14 (0.4), and 17 (0.20) were close to the detection limit in the acceptor compartment (Table S5, ESI†). This result indicated that the permeability of these compounds was comparable to the detection limit of low permeable standard atenolol. Conversely, parent molecule 6 was highly permeable. In summary, our data demonstrated that diffusion across cellular membranes of fluorescein-derivatives is strongly impaired in compounds 14–16.

Given the limited efficacy of the fluorescein-based probes to enter cells by diffusion, a new strategy was envisaged with nitrobenzofurazan (NBD) derivatives instead of fluorescein. In the absence of an extracellular receptor aiding in internalization, the use of a smaller and more neutral dye like NBD could be beneficial. To this end, 4-chloro-7-nitrobenzofurazan was modified with 2-bromoethylamine and subsequently reacted with 13 after a deprotection step to remove Boc to generate 18 (see ESI†). Internalization of compound 18 in LNCaP cells was then evaluated. Confocal images were taken after 1, 2 and 4 h incubation and compared with 16 that bears a fluorescein derivative. The retrieved images indicated that the fluorescent signal was modest, which suggests poor internalization of the NBD-labelled probes (Fig. S4, ESI†). Moreover, no improvements in the fluorescence intensity were observed relative to 16. The use of NBD in place of fluorescein was therefore an unsuccessful strategy to enhance dye diffusion and accumulation in cells. Altogether, these results highlight the absence of a receptor mediating the internalization process.

By considering the biophysical and cell-based experiments presented herein, one can determine the appropriateness of the site of conjugation in compound 6. The systematic exploration presented agrees with structure activity relationship studies present in the literature.11,18 Our findings also expose challenges and opportunities for forthcoming drug delivery systems based on BET protein inhibitors. Although these may be suitable per se as ligands or payloads for targeted constructs, the required derivatization and spacer length can strongly disrupt the physicochemical properties needed for cellular membrane permeation. These features are key if intracellular ligand recognition is responsible for the selectivity of the construct. In a broader context, our data highlights the importance of informed design and selection of linkers and payloads, which is often neglected.

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Conflicts of interest

R. T. and G. J. L. B. are co-inventors on a patent application.

Notes and references