X-RAY CRYSTAL STRUCTURES OF HITS FROM A FRAGMENT BASED DISCOVERY PROGRAM ON EPIGENETICS TARGET BRD3

PEAK PROTEINS

SYGNATURE discovery

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INTRODUCTION

Bromodomain-containing protein 3 (BRD3, also known as RING3L) like other members of the Bromodomain and Extra-Terminal motif (BET) family contains two tandem homologous bromodomains and an extra terminal motif. It functions by binding acetylated lysine residues on chromatin[1] and transcriptional regulators, exemplified by the role it plays in the regulation of transcription by promoting the binding of GATA1[2] (Figure 1). Due to this involvement in regulation, BET family members often play a role in several types of cancer[3]. BRD3 in particular is associated with a number of disease phenotypes. For example, depletion of BRD3 slows growth in cancer models including prostate cancer and medulloblastoma[4] and it presence has also been implicated in NUT midline carcinoma (NMT)[5].

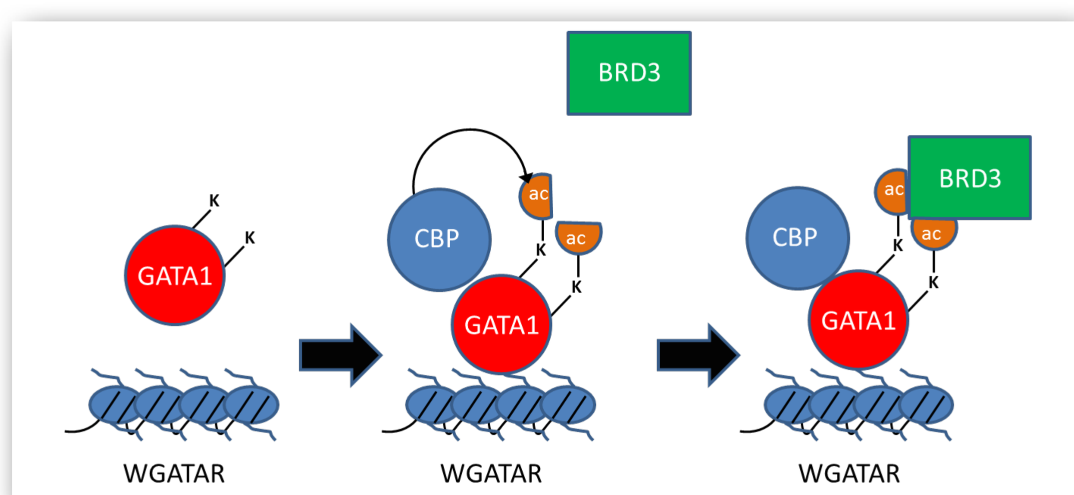


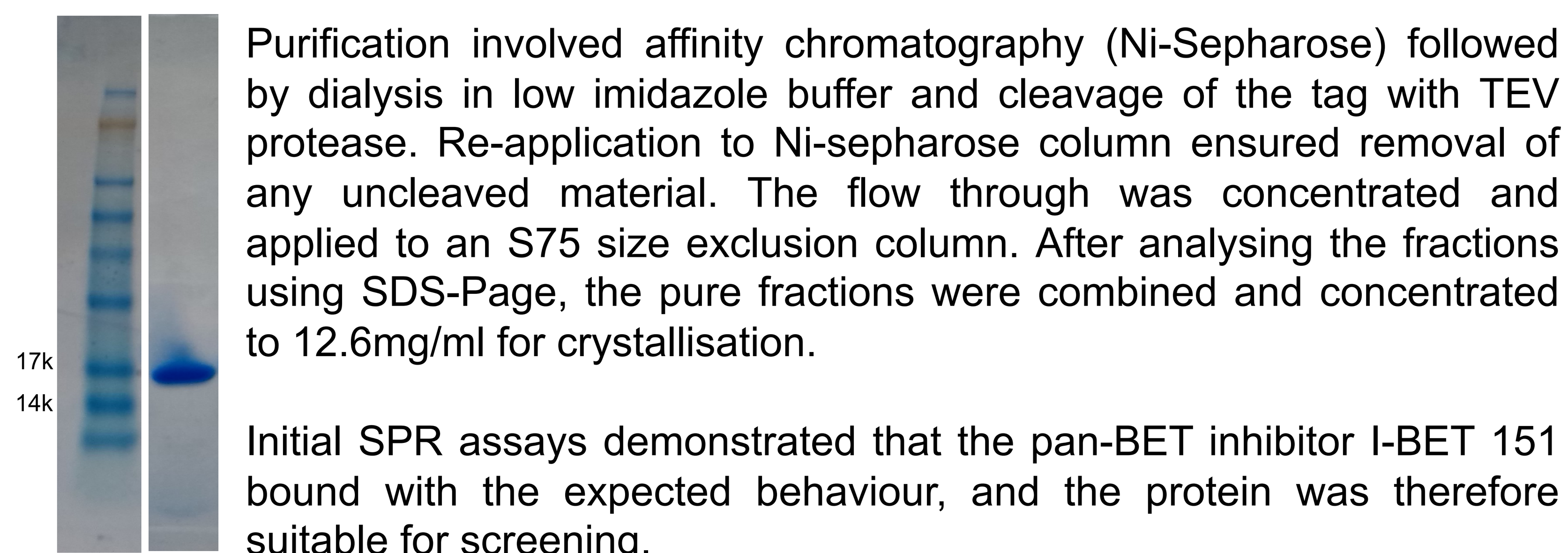
Figure 1: BRD3 binds acetylated lysine residues on chromatin and transcription regulators, such as GATA1. (Image adapted from reference 3).

While there are a number of available pan-BET inhibitors, the design of specific BRD3 inhibitors may lead to a beneficial clinical outcome with reduced off-target effects. However, bromodomains of the BET family have a high degree of structural similarity, especially in the acetylated lysine binding pocket, making the design of selective inhibitors problematic. BRD3 therefore represents an intriguing and challenging drug discovery target.

PROTEIN PRODUCTION

A cDNA clone of BRD3 domain 1 (BRD3D1) containing an N-terminal 6xHis-tag was obtained from the SGC[6] and expressed in *E. coli* (BL21 Gold).

Construct Sequence:
mhhhhhhssgvdlgtenlyfqsMPEVSNPSKPGRKTNQLQYMQNVVVKTLWKHQFAWPF
YQPVDAIKLNLPDYHKIIKNPMDMGTIKKRLENNYYWSASECMQDFNTMFTNCYIY
NKPTDDIVLMAQALEKIFLQKVAQMPQEE



Purification involved affinity chromatography (Ni-Sepharose) followed by dialysis in low imidazole buffer and cleavage of the tag with TEV protease. Re-application to Ni-sepharose column ensured removal of any uncleaved material. The flow through was concentrated and applied to an S75 size exclusion column. After analysing the fractions using SDS-Page, the pure fractions were combined and concentrated to 12.6mg/ml for crystallisation.

Initial SPR assays demonstrated that the pan-BET inhibitor I-BET 151 bound with the expected behaviour, and the protein was therefore suitable for screening.

Figure 1: SDS gel of purified BRD3 domain 1

FRAGMENT SCREENING

SURFACE PLASMON RESONANCE (SPR)

Fragment screening by SPR was carried out at Sygnature Discovery using a diverse in-house designed and synthesised library. The library has been clean screened to remove any PAINS (pan-assay interference compounds) or reactives and all fragments have been purity checked (> 95% purity) using NMR and LCMS. The library was designed using the 'rule of 3' as guidelines rather than applying strict cut-offs. Therefore, while the average properties fall within the 'rules', the chemical space is extended.

908 fragments in total were screened at 250 μM, 1% DMSO against BRD3/BD1, BRD4/BD1 (for selectivity/confirmation) and human carbonic anhydrase II (for specificity). This resulted in 275 actives that were further interrogated for sensorgram shape (i.e. square wave), effects on baseline and potential for superstoichiometric binding. Using these criteria the actives were reduced to 148. By performing concentration response curves to calculate K_D for each of these, it was found that 20 fragments had repeatable, saturating dose responses. Nine fragments were then chosen based on the reproducibility of the dose responses with solid stock for further characterisation by X-ray crystallography.

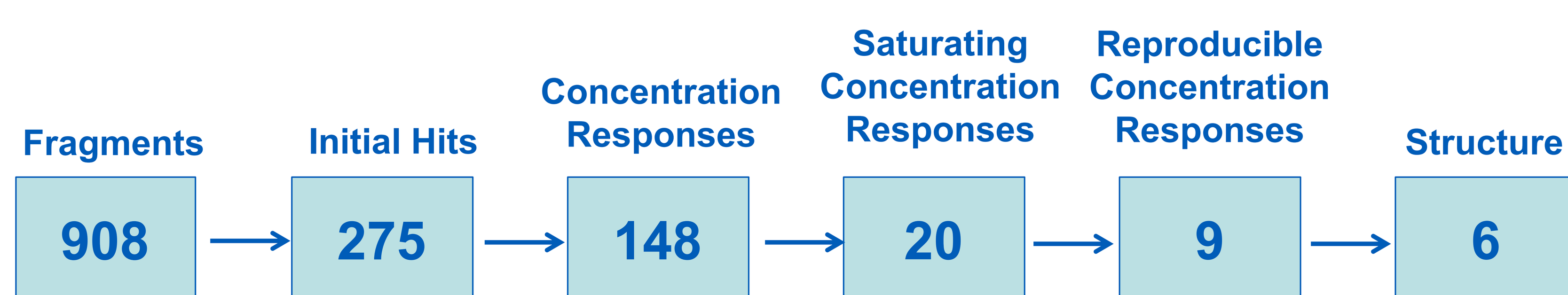


Figure 2: Of 908 fragments screen, 9 gave reproducible data from solid stock and 6 resulted in crystal structures.

CRYSTALLISATION

Crystals were grown in sitting drop MRC plates at 4°C. Drops contained 200nl protein and 200nl of 18-22% Peg3350, 200mM KSCN, 100mM Tris/HCl pH 8.4 to 8.8 with 10% glycerol[1]. The space group of the resulting crystals was $P2_12_12_1$ with unit cell a=50.8 b=61.8 c=84.1 $\alpha=\beta=\gamma=90.0°$

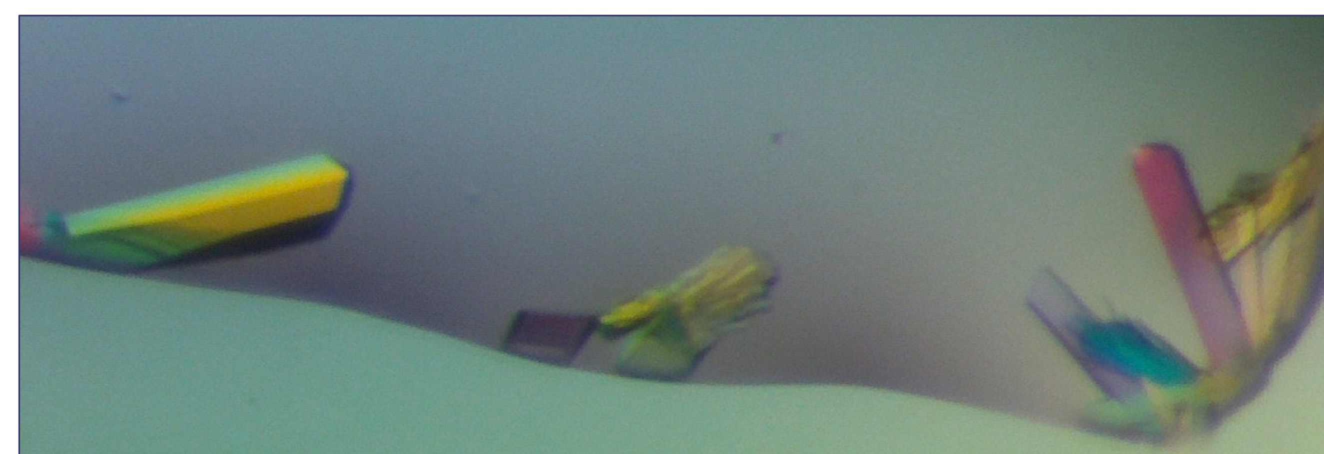


Figure 3: Crystals of BRD3 domain grown by sitting drop vapor diffusion

Fragments were dissolved in DMSO at 200mM (or 100mM for less soluble compounds). The apo crystals were transferred into a soaking solution (1:100 fragment stock:reservoir solution) and incubated overnight. Crystals were frozen in liquid nitrogen using 25% glycerol as a cryo protectant.

X-RAY STRUCTURES

X-ray diffraction data of all the soaked BRD3 crystals was collected at the Diamond Light Source on the I03 beamline and processed using DIALS[7]. The resolution of all processed data sets ranged from 1.4Å to 1.7Å. Initial models were based on the published PDB structure 2NXB while the resulting ligand bound complexes were modelled and refined using Coot[8] and REFMAC5[9] respectively. Initial electron density maps revealed unambiguous binding modes for all 6 structures.

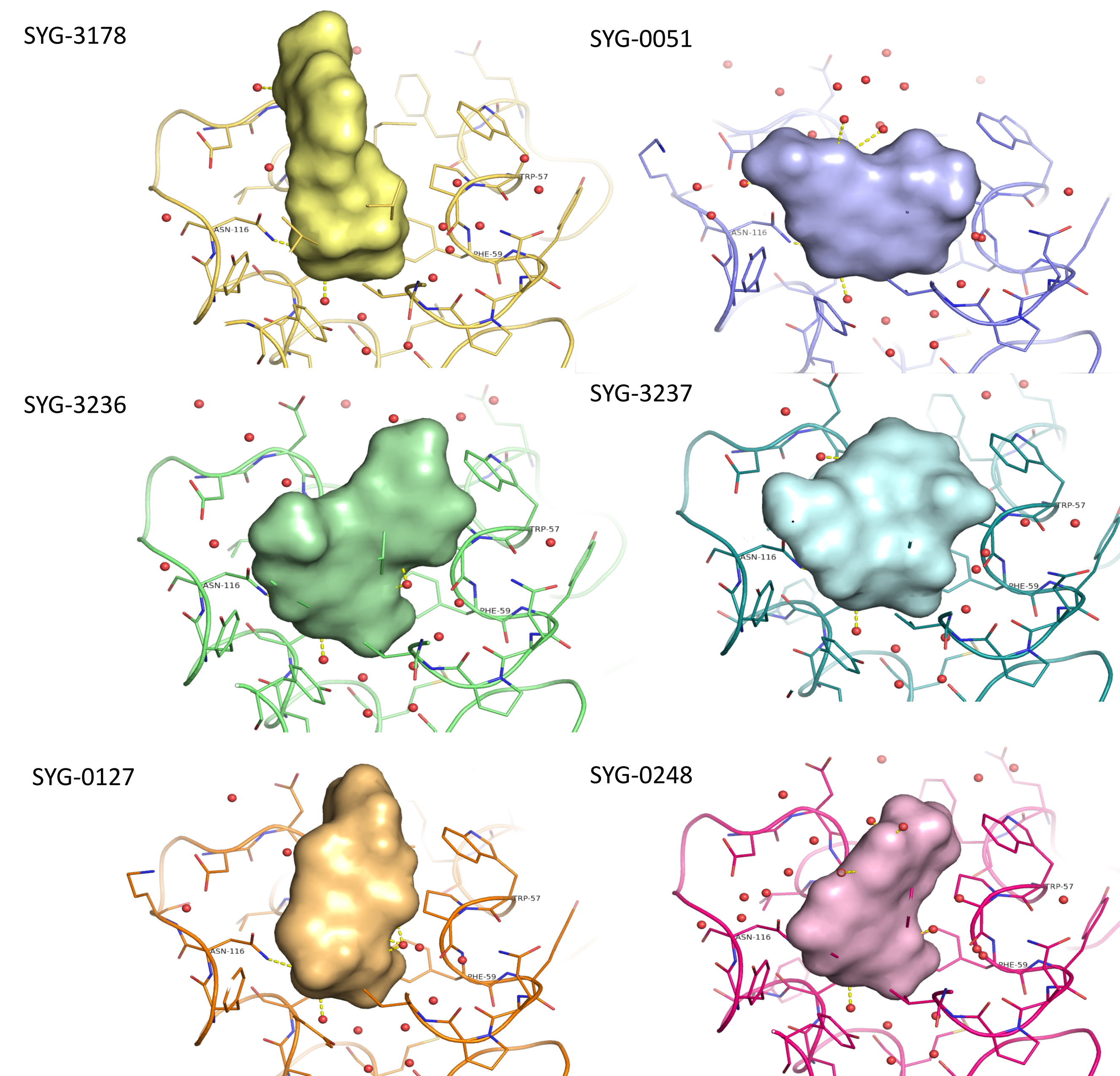


Figure 5: Six fragments bound to BRD3 BD1 as confirmed by X-ray crystallography. The fragments are represented as molecular surfaces that were generated using PyMOL.

All fragments were observed to bind as expected in the acetyl lysine binding site and to form hydrogen bonds with both the conserved Asn116 and water network within the pocket. Two fragment analogues (SYG-3236 and 3237) that gave comparable K_D by SPR (42 μM & 35 μM respectively) were shown to bind BRD3 BD1 in a similar manner (Figure 5). Another fragment, SYG-0127, was the most potent fragment tested having a K_D <10 μM. This potency most likely derives from its 5 water mediated interactions, the highest number found in these structures (Figure 5).

CONCLUSIONS

The high resolution X-ray crystal structures of BRD3 domain 1 in complex with hits from a SPR fragment screening program were solved to high resolution. Of 908 fragments screened, nine were found to bind with K_D ranging from <10 μM to 250 μM and six were confirmed by crystallography.

This initial data can be used as a potential starting point for a new drug discovery program, to include hit expansion, refinement and characterisation of selective compounds in biological assays. By using these fragments as starting points to develop tool ligands, BRD3 involvement in disease phenotypes could be further interrogated, shedding more light on the role of this under-investigated epigenetic target.

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