Supporting Information

Fragment-Based Screening of the Bromodomain of ATAD2

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EXPERIMENTAL

Protein Expression and Purification – The codon-optimized expression plasmid encoding residues 979-1108 of human ATAD2 (UniProt ID Q6PL18) was purchased (Structural Genomics Consortium, Oxford) and transformed into *Escherichia coli* BL21 (DE3) GOLD (Stratagene) using kanamycin for selection.¹ To facilitate purification, this plasmid has both a N-terminal His tag and TEV cleavage site. A single colony was selected and used to inoculate a 200-mL overnight culture of LB-Kan grown at 37 °C. This culture was used to inoculate a 10-L fermentation culture (BioFlo 415, New Brunswick Scientific). Unlabeled ATAD2 for X-ray crystallography purposes was overexpressed in LB media, while isotopically labeled (¹⁵N) ATAD2 for NMR spectroscopy was overexpressed in M9 minimal media containing ¹⁵NH₄Cl (Cambridge Isotope Laboratories) as the sole nitrogen source. Protein expression was carried out at 18 °C overnight following addition of 0.5 mM IPTG once cultures reached OD₆₀₀ of 1.0-1.5 for LB or 0.7-1.0 for M9 media. Cultures were briefly chilled from 37 to 18 °C prior to addition of IPTG. Cells were harvested after 16 h by centrifugation and frozen at -80 °C. The cell pellet was resuspended to homogeneity in Lysis Buffer (50 mM HEPES, 500 mM NaCl, 3 mM imidazole, 5% glycerol, 3 mM BME, pH 7.5) at approximately 10 mL/g of pellet and homogenization at 4 °C was used to lyse the cells (APV-2000, APV). The lysate was cleared by centrifugation at 14,000 rpm for 1.5 h, followed by filtration through 0.45 µm filters. Cleared lysate was loaded on a 150-mL Ni-Probond column (Invitrogen) equilibrated in Lysis Buffer with an Akta Purifier. ATAD2 was eluted from the column using a gradient from 0-75% Elution Buffer (50 mM HEPES, 500 mM NaCl, 350 mM imidazole, 5% glycerol, 3 mM BME, pH 7.5). Fractions from the column were pooled, concentrated in a stirred ultrafiltration cell (Amicon, Millipore), and dialyzed against Lysis Buffer overnight using 6-8 kDa MWCO dialysis tubing (Spectrum Labs). TEV protease was added to the fusion protein at a 1:10 molar ratio (TEV:ATAD2) to remove the purification tag with stirring at 4 °C overnight. The purification tag and TEV protease were removed from free ATAD2 by use of a 120-mL Ni-NTA subtractive column (Qiagen). Fractions were pooled, concentrated, and exchanged into NMR /X-ray Buffer (50 mM HEPES, 150 mM NaCl, 2 mM DTT, pH 7.5) via dialysis for NMR studies. For X-ray crystallography, free ATAD2 was further purified by loading on a 320-mL Superdex 75 column (GE Healthcare) equilibrated in NMR/X-ray Buffer. All protein was stored at -20 °C until needed for experimentation.

NMR Spectroscopy – Nuclear magnetic resonance (NMR) screening experiments were performed at 25 °C using a Bruker DRX 500 MHz spectrometer equipped with a 5 mm single-axis x-gradient cryoprobe and a Bruker Sample Jet. Gradient enhanced, two-dimensional ^1H - ^{15}N heteronuclear multiple-quantum coherence spectra (SOFAST-HMQC) spectra were recorded on ATAD2 (16 scans, ~7 min).² Spectra were processed and analyzed using Topspin, version 3.1 (Bruker Biospin). Our in-house fragment library of ~13,800 compounds was screened as mixtures of 12 fragments in a 96-sample format using ~70 μM of ^{15}N -labeled ATAD2, 800 μM of each fragment, and 5% DMSO- d_6 for spectrometer locking purposes. Fragment library composition is described elsewhere.^{3, 4} Hit mixtures were identified by comparing the chemical shifts of backbone resonances to a ligand-free ATAD2 reference spectrum. Because fragments were screened as mixtures, deconvolution of hit mixtures was necessary to isolate individual hit fragments. Deconvolution was accomplished by screening each twelve-compound mixture hit as a series of twelve singleton samples.

SOFAST-HMQC titration experiments were used to quantify the binding affinity of the best hits identified from the screen. The change in ^1H chemical shift of backbone resonances upon stepwise addition of fragment was measured and fit by an in-house script.⁵

X-ray Crystallography – Unlabeled ATAD2 was concentrated to 10-12 mg/mL and passed through a 0.22 μm eppendorf tube filter. A screen around the published crystallization conditions for ligand-free ATAD2 was used to generate crystals for fragment soaking experiments.¹ Ligand-free ATAD2 crystals grew at 4 and 18 °C in 1-3 days (0.1 M Bis-Tris pH 5.5, 2.5 M $(\text{NH}_4)_2\text{SO}_4$). Ligand-free crystals were soaked in mother liquor supplemented with 20 mM fragment (**1** and **12**) overnight at 18 °C. Soaked crystals were cryoprotected in mother liquor supplemented with saturated $\text{Li}_2\text{SO}_4 \cdot \text{H}_2\text{O}$ prior to freezing in liquid nitrogen. Co-crystals of ATAD2 bound to fragment were generated at 4 and 18 °C in 1-3 days by addition of 8 mM fragment (**5**) to ligand-free ATAD2 (0.1 M Bis-Tris pH 6.0, 2.2 M $(\text{NH}_4)_2\text{SO}_4$, 10% glycerol). X-ray data was collected on the Life Sciences Collaborative Access Team (LS-CAT) Sector-21 beamlines at the Advanced Photon Source (APS), Argonne National Labs. Data was indexed, integrated, and scaled with HKL2000.⁶ Molecular replacement with Phaser⁷ was accomplished using Phenix⁸ and the ligand-free ATAD2 structure determined by the Structural Genomics Consortium (PDB ID 3DAI) as the search model.¹ Structure refinement was accomplished with Phenix and included several rounds of manual model building with COOT.⁹ Figures have been prepared using PyMOL.¹⁰

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Table S1. Residues directly interacting with inhibitors in BRD4 compared to those positions when aligned with ATAD2

BRD4 (PDB ID 3UVW)	ATAD2 (PDB ID 4QUU)
W81	V1008
P82	No match in alignment
L92	V1018
L94	No match in alignment
Y139	Y1063
N140	N1064
I146	I1074

Figure S1. View of residues within the Kac binding site of BRD4 that interact with diacetylated peptides. The two Kac residues of histone-derived peptide (H4Kac5Kac8) are shown as teal sticks (PDB ID 3UVW).

Figure S2. ^1H - ^{15}N HMQC spectral overlays. The reference ligand-free ATAD2 spectrum (black) shown overlaid with ATAD2 in the presence of (A) fragment **1**, (B) fragment **5**, and (C) fragment **12** (all in red). In panels (B-C), green circles indicate residues whose chemical shift may report on ligand binding deeper inside the Kac binding pocket, although backbone resonance assignments are unknown.

Figure S3. (A-C) Fragments **1**, **5**, and **12** modeled into Fo-Fc omit maps (contoured at 3, 2, and 2 σ respectively). (D-F) Stereo images of fragment **1**, **5**, and **12** bound in the Kac binding site. Residues shown as sticks, water molecules, and polar contacts are all as shown in Figure 3 of the text (PDB IDs 4TYL, 4TZ2, and 4TZ8).

Figure S4. Water molecule displacement by **5** and **12**. (A) By comparing crystallographic water molecules in the Kac binding pocket in the structures with **5**, thymidine, and **12** bound, it is concluded that **5** displaces a sum of 4 water molecules. Molecules are shown as follows: **5** (magenta sticks and water molecules), thymidine (yellow sticks and water molecules), and **12** (cyan sticks and water molecules) (PDB IDs 4TZ2, 4QSV, and 4TZ8). (B) By comparing crystallographic water molecules in the presence of **12**, **1**, and thymidine, it is concluded that **12** displaces one water molecule. Molecules are shown as follows: **1** (green sticks and water molecules), **12** (cyan sticks and water molecules), and thymidine (yellow sticks and water molecules) (PDB IDs 4TYL, 4TZ8, and 4QSV). Yellow circles indicate waters that are shared between the overlaid complexes. Dashed red circles indicate waters that are displaced by the binding of **5** or **12**.

Figure S5. Chemical structures of (A) thymidine and (B) 1-methylquinolin-2-one.