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Observed bromodomain flexibility reveals histone peptide- and small molecule ligand-compatible forms of ATAD2A

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Abstract

Preventing histone recognition by bromodomains emerges as an attractive therapeutic approach in cancer. Overexpression of ATAD2A in cancer cells is associated with poor prognosis making the bromodomain of ATAD2A a promising epigenetic therapeutic target. In the development of an invitro assay and identification of small molecule ligands, we conducted structure-guided studies which revealed a conformationally flexible ATAD2A bromodomain. Structural studies on apo-, peptide and smallmolecule-ATAD2A complexes (by co-crystallization) revealed the bromodomain adopts a “closed”, histone-compatible conformation, and a more “open” ligand-compatible conformation of the binding-site respectively. An unexpected conformational change of the conserved asparagine residue plays an important role in driving the peptide-binding conformation remodelling. We also identified dimethylisoxazole-containing ligands as ATAD2A binders which aided in the validation of the invitro screen and in the analysis of these conformational studies.

Keywords

Cancer target; Epigenetics; ATAD2A bromodomain; protein flexibility; H4K5ac peptide

Introduction

The epigenetic regulation of chromatin structure and function is critical for transcriptional control, cell identity and development. Several groundbreaking studies have demonstrated the importance of post-translational modifications of histones, proteins and DNA for the

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epigenetic control of gene expression [1-3]. Notably, the identification of chromatin-associated proteins that catalyze, recognize and remove these chemical markers has led to the concept of the 'histone code' and the idea of writers, readers and erasers [4]. These proteins work in concert with transcription factors to control fundamental cellular processes including proliferation, development, differentiation and genome integrity. Consequently, disruption of epigenetic control can lead to aberrant gene expression and human disease. Indeed, growing genomic evidence links mutations, amplifications, deletions and rearrangements of genes encoding epigenetic regulators to the development of human cancer. Consequently, therapeutic targeting of chromatin-associated proteins is a growing area of drug discovery. In fact, the first epigenetic-based therapies for cancer treatment which act globally on these post-translational processes (i.e. histone deacetylase inhibitors and inhibitors of DNA methylation) have been approved. However, preclinical evidence suggests that targeting specific writers (such as the histone methyl transferases EZH2 and DOT1L) or reader proteins (such as the BET family of bromodomains) may be beneficial in defined patient populations and select tumor types. For example, DOT1L may be useful in 11q23-related leukemias associated with MLL-rearrangement and inhibition of the reader protein BRD4 may prove beneficial in certain tumor types as BRD4 appears critical for MYC transcriptional pathways [5].

ATAD2A is an epigenetic regulator that has been highlighted as a promising target for anti-cancer therapeutic intervention [6]. Studies revealed that high ATAD2A expression is significantly associated with poor survival and progression of cancers including lung and prostate [6, 7]. Copy-number gain and co-amplification of ATAD2A with c-MYC is also observed across many tumors and it is the most significantly upregulated bromodomain-containing gene across the TCGA tumor atlas. In addition, ATAD2A has been characterized as a transcription co-regulator of the hormonally-controlled developmental process interacting with the estrogen receptor (ER α) and the androgen receptor (AR) in breast and prostate cancer, respectively [8, 9]. Specifically, ATAD2A expression has been hypothesized to lead to an amplification loop in these cancers driving highly proliferative transcriptional programs including EF2 and cMYC. Finally, numerous knock-down studies of the ATAD2A protein in cancer (and normal) cells suggest a potential role in cell proliferation and transformation [6, 8, 9].

The domain architecture of ATAD2A includes an AAA ATPase domain and a bromodomain, which could both be targeted by small molecule inhibitors [10]. While these inhibitors of AAA ATPases have been reported for dynein [11] and p97/VCP [12, 13], this enzyme class is challenging from an inhibitor selectivity/specificity point of view. In contrast, selective bromodomain inhibitors have emerged with selective BET-family bromodomain inhibitors showing promising preclinical [5] and clinical activity [14, 15]. Bromodomains are structurally and evolutionary conserved ~110 amino acid modules present in a large number of chromatin-associated proteins (including ATPase-dependent chromatin remodeling complexes and in nearly all nuclear histone acetyltransferases). For the majority of bromodomains, there is little definitive knowledge about their cognate sequences and physiological recognition motifs.

The purpose of our study was to identify chemical probes in order to develop ligands of the ATAD2A bromodomain. We hypothesized that we could obtain inhibitors by targeting the acetyllysine binding pocket, as reported for other bromodomains [16-18]. However, prior to embarking on a medicinal chemistry effort, structural biology was used to identify acetyllysine pocket binders, such as histone peptides or small molecule probes, in order to characterize their binding mode at the molecular level. To do this we first identified the H4K5 acetyl peptide (H4K5ac) as an active site binding partner using biophysical and X-ray crystallographic approaches. We focused on co-crystallization rather than soaking technique, and extensive characterization of the H4K5ac complex revealed an unexpected conformational change accompanying the peptide recognition and provided an undescribed ATAD2A form in a closed state. Including structural data from a novel empty form of ATAD2A bromodomain we compared a total of 6 states, confirming experimentally the evidence of protein dynamics that has been described by molecular dynamics simulation [19], and providing a snapshot of the mechanism for peptide recognition and accompanying loop flexibility. With the recent identification of ATAD2A small-molecule binders by our lab and others [20, 21], our study highlights that conformational flexibility of the bromodomain of ATAD2A has implications for histone-peptide recognition towards the development of ATAD2A bromodomain inhibitors.

Material and Methods

Peptides used for α -screen assay, direct binding and crystallization studies

Biotinylated and non-biotinylated peptides used for α -screen assay; ITC and crystallization were purchased from Anaspec: H2AK36Ac (19-38)-biotin, SRAGLQFPVGRVHRLLRK(Ac)GNK-biotin; H2BK85Ac (81-93)-biotin, AHYNK(Ac)RSTITTSREK-biotin; H3K14ac (1-21)-biotin, ARTKQTARKSTGGK(Ac)APRKQLAGGK-biotin; H3K56Ac (44-57)-biotin, GTVALREIRRYQK(Ac)SK-biotin; H4K5ac (1-25)-biotin, SGRGK(Ac)GGKGLGKGGAKRHRKVLRDNGSGSK-biotin; H4K8Ac (1-25)-biotin, SGRGKGGK(Ac)GLGKGGAKRHRKVLRDNGSGSK-biotin; H4K12Ac (1-25)-biotin, SGRGKGGKGLGK(Ac)GGAKRHRKVLRDNGSGSK-biotin; H4K5/8/12/16-Ac4 (1-25)-biotin, SGRGK(Ac)GGK(Ac)GLGK(Ac)GGAK(Ac)RHRKVLRDNGSGSK-biotin; H4 (1-25)-biotin, SGRGKGGKGLGKGGAKRHRKVLRDNGSGSK-biotin; H4(1-20), SGRGKGGKGLGKGGAKRHRK; H4K5ac(1-20), SGRGK(Ac)GGKGLGKGGAKRHRK; H3K14ac (1-21), ARTKQTARKSTGGK(Ac)APRKQLAGGK.

Plasmids, Cloning, Site-directed Mutagenesis

The *Escherichia coli* strains BL21 (DE3) star were purchased from Invitrogen. The ATAD2A (EC 3.6.1.3) bromodomain coding sequence was amplified using a pNIC28-Bsa4 vector providing by the structural genomics consortium. Wild-type and mutated genes were sequenced, and the corresponding plasmids were then used for transforming the *E. coli* strain BL21 (DE3) star for protein expression. The transformants were grown at 22 °C in LB medium in the presence of kanamycin at 50 mg/ml during 24 hours. The expression of both recombinant proteins was then induced by the addition of 1 mM isopropyl- β -D-1-thiogalactopyranoside, and growth was continued for 24 hours at 16 °C. The cells were then

pelleted by centrifugation and served as the source for protein purification. Soluble protein was purified using Ni-NTA (Qiagen) gravity flow affinity chromatography followed by TEV cleavage and size exclusion chromatography. The protein was concentrated to 12–14 mg/ml in 25 mM HEPES, pH 7.5; 150 mM NaCl, and 10 mM DTT column buffer.

ATAD2A AlphaScreen Assay

Recombinant human His-tagged ATAD2A (produced in-house), biotinylated H4 K5Ac (1-25) peptide, and test compound were added to a 384-well OptiPlate (Perkin Elmer) and incubated at room temperature for one hour. The assay buffer consisted of 50 mM HEPES, pH 7.5, 100 mM NaCl, and 0.12 mM Triton X-100. Final concentrations for this reaction were as follows: 100 nM His-ATAD2, 100 nM peptide, variable concentrations of compound (3-fold serial dilutions), and 1% (v/v) DMSO. Streptavidin donor beads and nickel chelate acceptor beads, both from a Perkin Elmer AlphaScreen Histidine Detection Kit, were then added to the Optiplate to a final concentration of 10 µg/mL each. Following a 2 hour incubation at room temperature, the microplate was read on an Envision Plate Reader (Perkin Elmer). Percent of control (POC) values were calculated from the following formula: $\text{POC} = \frac{\text{sample signal} - \text{average background signal}}{\text{average maximum signal} - \text{average background signal}} \times 100$. The average maximum signal was obtained from wells containing all assay components except test compound. The average background signal pertained to wells with all assay components except ATAD2A and test compound.

Measurement of small molecule K_d by DiscoverX and Ligand efficiency calculation

Measurement of affinities by the DiscoverX competitive displacement assay has been assessed as previously described [22]. Ligand efficiencies (LEs) were calculated using the following formula: $\text{LE} = 1.37 \times (\text{pK}_d / \# \text{ of heavy atoms})$ as previously described [23].

Crystallization

Crystallization conditions for the double mutant alanine Y1063A-N1064A were similar to the conditions reported by the structural genomic consortium for WT-ATAD2A [24]. Protein crystallized at 4°C by vapor diffusion in a hanging drop with a crystallization buffer containing 2.0 M ammonium sulfate, 0.1 M Bis-Tris pH 5.5. Initial co-crystallization conditions of WT-ATAD2A in complex with H3K14ac and H4K5ac peptides were found by using a sitting drop-based sparse-matrix screening strategy at 4°C. Peptide solution stocks were diluted in the protein column buffer at 5 mM. Protein at 8–10 mg/ml was incubated with H3K14ac or H4K5ac peptide to reach a 1:3 ratio. For both complexes, the best crystals were obtained after 3–5 days of equilibration using a sitting drop method by mixing 1 µl of the peptide-protein solution with an equal volume of the reservoir equilibrated over 0.3 ml of the crystallization buffer at 4°C. Co-crystals with H4K5ac were obtained at 4°C using a crystallization buffer containing 0.1 M ammonium acetate, 100 mM Bis-Tris, pH 5.5, 15–20% w/v polyethylene glycol 10000. Co-crystals with H3K14ac were obtained at 4°C using a crystallization buffer containing 2.0 M ammonium sulfate, 100 mM Tris, pH 8.5. ATAD2A crystals showing the double conformation of N1064 were obtained at 4°C using a crystallization buffer containing: 0.2 M ammonium sulfate/ 0.1 M Bis-Tris pH 5.5/ 25% w/v PEG3350. For cryoprotection, all crystals were transferred in their relative crystallization

buffer supplemented by 20% glycerol. Co-crystallizations with compound **1** and compound **2** were performed at room temperature with an hanging drop method by mixing an equal volume of the protein incubated with the compound of interest (1 mM final concentration 1% DMSO) and the solution reservoir containing 2.1-2.4 M ammonium sulfate, 0.1 M buffer (Bis-Tris pH 5.2-5.7), 10% Glycerol. In order to get 100% of occupancy in the binding site, co-crystals with compounds were next soaked for 24 hrs in a pre-equilibrated hanging drop containing 2.0 M ammonium sulfate, 0.1 M buffer Bis-Tris pH 5.5, 10% glycerol and the compound of interest at 10 mM final concentration.

Crystallographic Studies

All X-ray diffraction data sets were collected from frozen single crystals at the Advanced Light Source (Berkley, CA, USA, beamline 8.3.1) and processed with the programs ELVES [25] and/or MOSFLM, SCALA, and TRUNCATE from the CCP4 program suites [26]. Molecular replacement solutions were obtained using the BALBES molecular replacement pipeline [27] and the crystal structure PDB code 3DAI [24]. Iterative model rebuilding and refinement were performed by using the program COOT and REFMAC5 [28, 29]. Figures of the different structures of ATAD2A were generated using PyMOL (DeLano Scientific).

Isothermal titration calorimetry

ITC measurements were carried out at 10 °C using the nano ITC (TA instruments) with a stirring of 250 rpm. ITC titrations were performed with one 0.2- μ l injection of peptide, followed by 20 consecutive injections of 2.0 μ l of the peptide with injection durations of 8- and 240-s intervals between injections. The sample chamber was filled with 100 μ M of wild-type or mutants ATAD2A protein. Solution stocks of peptides were prepared at 5 mM using the column buffer used during the protein purification. Solutions of peptide titrants were next freshly prepared by diluting them to reach a 1.5 mM final concentration. Titrant solutions and protein were centrifuged for 10 min at $14,000 \times g$, and supernatants were loaded into the syringe and cell, respectively. The final molar ratio of ligand:protein exceeded 2.5. The heat released was measured following each injection. Data from the experiment were analyzed with Nanoanalyze software (TA instrument) supplied by the manufacturer.

Synthetic route to Compound 2

Methyl 3-amino-5-(3,5-dimethylisoxazol-4-yl)benzoate—To a solution of (3,5-dimethylisoxazol-4-yl)boronic acid (123 mg, 0.869 mmol) and methyl 3-amino-5-bromobenzoate (200 mg, 0.869 mmol) in dimethoxyethane was added sodium carbonate powder (193 mg, 1.83 mmol) in water (1 mL) and palladium tetrakis (triphenylphosphine) catalyst (30 mg, 3 mol%). The reaction mixture was degassed by sparging with a stream of nitrogen and then refluxed for 16 hrs. The cooled reaction mixture was partitioned between water and ethyl acetate, the organic layer was dried over sodium sulfate, filtered, concentrated and then purified by flash chromatography (1:1 ethyl acetate/hexanes) to give methyl 3-amino-5-(3,5-dimethylisoxazol-4-yl)benzoate as a pale-yellow solid (190 mg, 89%). MS (ESI): m/z calcd. for $(C_{13}H_{14}N_2O_3 + H)^+$ 247.1, found 246.8. 1H NMR (600

MHz, d₆-DMSO) δ 7.20 (s, 1H), 7.01 (s, 1H), 6.78 (s, 1H), 5.54 (s, 2H), 3.82 (s, 3H), 2.38 (s, 3H), 2.20 (s, 3H).

Methyl 3-(3,5-dimethylisoxazol-4-yl)-5-(phenylsulfonamido)benzoate

(Compound 1)—A solution of methyl 3-amino-5-(3,5-dimethylisoxazol-4-yl)benzoate (30 mg, 0.122 mmol) and pyridine (0.029 ml, 0.365 mmol) in dichloromethane (1 ml) was treated with benzenesulfonyl chloride (22 mg, 0.122 mmol) and the reaction mixture stirred at ambient temperature for 1 day. The reaction mixture was concentrated then purified by mass-directed prep-HPLC (mobile phase: A = 0.1% TFA/water, B = 0.1% TFA/acetonitrile; Gradient: B = 30% - 70% in 12 min; Column: C18) to give methyl 3-(3,5-dimethylisoxazol-4-yl)-5-(phenylsulfonamido)benzoate as a white solid (14 mg, 30%). MS (ESI): *m/z* calcd. for (C₁₉H₁₈N₂O₅S + H)⁺ 387.1, found 386.8. ¹H NMR (600 MHz, CDCl₃) δ 7.83 (d, *J* = 7.5 Hz, 2H), δ 7.67 (d, *J* = 1.9 Hz, 2H), 7.58 (t, *J* = 7.8 Hz, 1H), 7.48 (t, *J* = 8.0 Hz, 2H), 7.29 (t, *J* = 1.9 Hz, 1H), 7.20 (s, 1H), 3.92 (s, 3H), 2.34 (s, 3H), 2.19 (s, 3H).

3-(3,5-Dimethylisoxazol-4-yl)-5-(phenylsulfonamido)benzoic acid (Compound 2)

(Compound 2)—A solution of methyl 3-(3,5-dimethylisoxazol-4-yl)-5-(phenylsulfonamido)benzoate (14 mg, 0.036 mmol) in water (0.3 ml), methanol (0.3 ml) and THF (0.3 ml) was treated with lithium hydroxide hydrate (3.0 mg, 0.072 mmol). The reaction mixture was heated to 50 °C for 4 h. The cooled reaction mixture was then acidified with aq. HCl (0.012 ml, 0.12 mmol) and purified by prep-HPLC (mobile phase: A = 0.1% TFA/water, B = 0.1% TFA/acetonitrile; Gradient: B = 20% - 50% in 12 min; Column: C18) to give compound 2 as a white solid (5 mg, 37 %). MS (ESI): *m/z* calcd. for (C₁₈H₁₆N₂O₅S + H)⁺ 373.1, found 373.4. ¹H NMR (600 MHz, d₆-DMSO) δ 13.20 (br-s, 1H), 10.66 (s, 1H), 7.80 (d, *J* = 7.8 Hz, 2H), 7.68 (s, 1H), 7.64 (t, *J* = 7.6 Hz, 1H), 7.58 (t, *J* = 7.6 Hz, 2H), 7.54 (s, 1H), 7.26 (s, 1H), 2.31 (s, 3H), 2.11 (s, 3H).

Quantification of the relative movement in the ZA loop

The relative position of V1018 has been used to quantify the ZA loop movement compare to the apo structure of reference PDB ID 3DAI. The relative distance between the C_{γ2} of V1018 and the atom C_γ from the acetyllysine side chain ($d_{\text{dist.}} = d \text{ V1018 (C}_{\gamma 2}) - \text{K5Ac (C}_{\gamma})_{\text{structure}} - d \text{ V1018 (C}_{\gamma 2}) - \text{K5 (Ac) (C}_{\gamma})_{\text{reference}}$) has been measured for each state. Positive differences indicate wider conformations, and negative differences indicate narrow conformations.

PDB accession codes

Atomic coordinates and structure factors have been deposited in the protein data bank under the accession code PDB ID: 4TT2, PDB ID: 4TT4, PDB ID: 4TT6, PDB ID: 4TTE, PDB ID: 4TU4, PDB ID: 4TU6.

Results

ATAD2A bromodomain recognizes H4K5ac peptide

In the search for chemical probes able to disrupt the ATAD2-acetylated histone complex, we sought to develop a high-throughput bromodomain histone peptide displacement assay. The

ATAD2A bromodomain is an acetyllysine reader, and histone interactions have been reported with H4K5ac or H3K14ac peptides based on co-immunoprecipitation studies in mouse and human cells, respectively [7, 30]. However, to our surprise such interactions have not been confirmed from panning of histone peptide panels by peptide array [24]. We used a high-sensitivity AlphaScreen assay widely used for bromodomains [31, 32] to screen for acetylated histone peptides. Interactions are detected by using a chemiluminescent readout triggered by the close proximity between a donor-bead coated peptide and an acceptor-bead coated protein. We screened a panel of histone peptides and characterized H4K5ac as a specific acetyllysine binder with a K_d of 22 μ M (Supplementary Fig. 1a, b). Despite the previous report of H3K14ac recognized by ATAD2A, we were surprised to find it did not respond in our AlphaScreen assay. We evaluated the structural basis for H4K5ac recognition by ATAD2A, and investigated the H3K14ac binding mode using X-ray crystallography. The assay development and the acetyllysine binding validation studies are documented in Supplementary Note 1 and 2, Supplementary Table 1 and Material and Methods sections.

Structural basis of H4K5ac readout by ATAD2A

The ATAD2A bromodomain contains a canonical left-handed four-helix bundle (α Z, α A, α B and α C), topped with 2 loops; ZA and BC loops (Fig. 1a). The acetyllysine binding pocket is described structurally by a cavity created with 2 helices (α B and α C) and the ZA and BC loops. Specific features of ATAD2A reported in Figure 1a highlight the presence of a specific RVF shelf and proline residues located in the loops that may confer a substantial degree of flexibility to the structure.

Following the submission of our manuscript, a group from the structural genomic consortium identified H4K5ac peptide as an ATAD2A binder. Despite packing constraints, an X-ray structure obtained by peptide soaking confirmed the interaction [20]. Because recent molecular dynamics studies suggested some flexibility of the ZA and BC loop on bromodomains [19], in the present study we prioritized a co-crystallization strategy. We identified alternative crystallogenes conditions and obtained the complex by co-crystallization. This complex allowed us to improve our knowledge of the H4K5ac readout and demonstrated that the peptide binds to the acetyllysine-binding site, as illustrated by the Fo-Fc omit map (Supplementary Fig. 2a). As seen for the recent complex obtained by soaking [20], the polypeptide follows the groove created by the ZA and BC loops. In our co-crystal structure residues 1-SGRGK_{ac}G-6 were defined, allowing us to determine the molecular basis of the recognition. A summary of data collection and refinement statistics has been reported in Supplementary Table 2.

Both, hydrophobic contacts and hydrogen bonds contribute to the peptide readout. Five hydrophobic contacts, involving residues from the ZA loop (V1008, V1013, V1018, Y1063) and helix α C (I1074), were found in the acetyllysine binding pocket (Fig. 1b). Notably, at the bottom of the cavity, all the hydrophobic interactions did not induce any side chain rearrangements despite a narrow environment. These interactions engaged the sp^2 hybridized carbon of the acetyllysine which interacts with both the ZA loop and the helix α C (through residues V1013 and I1074, respectively), and the sp^3 hybridized carbon which makes contact with the RVF shelf (V1008). Two additional hydrophobic contacts stabilize the C $_{\gamma}$

of the acetyllysine side chain in the upper part of the cavity (V1018 and Y1063) (Fig. 1b). Taken together, these observations show that the hydrophobic contacts surrounding the acetyllysine cavity play a key role in peptide recognition.

In addition to hydrophobic contacts, two residues from the peptide at position 3 and 5 (R3 and K5ac) form hydrogen bonds and contribute to the specific nature of binding. (Fig. 1c). We observe the common signature of interactions of acetyllysine peptides to bromodomains [18] in which ATAD2A acetyllysine interacts directly with the conserved asparagine (N1064) and engages the ZA loop and α B helix via a series of hydrogen bonds through water molecules (Fig. 1c). The R3 side chain interacts with 2 carbonyls of the protein's backbone at the top of α B helix (E1062 and Y1063) (Fig. 1c). Taken together, these observations highlight the requirement of R3 for the specificity of the acetyllysine peptide recognition.

ATAD2A loop motion facilitates histone recognition leading to a closed state

Superimposition of the apo [24] and our peptide bound structures reveals evidence of both ZA and BC loop flexibility which accompanies the recognition of H4K5ac (Fig. 2a). Computational studies have suggested apparent bromodomain flexibility [19, 33, 34], however no loop rearrangements have been described experimentally in the bromodomain family upon histone binding, describing an additional mode of molecular recognition for bromodomains. Thus, we decided to compare the movement of each loop in detail.

In the peptide bound complex, the ZA loop movement shrinks the acetyllysine binding site allowing V1013 and V1018 to shift inward toward the cavity (by 1.1 at the bottom and 1.7 Å, respectively; Fig. 2b). The ZA loop movement is mediated by 2 prolines (P1012 and P1028) which act as hinges (Fig. 2b) which agrees with Pizzitutti et al. [33] who showed that P371 of the Gcn5p bromodomain (corresponding to P1012 here) plays a key role in the molecular recognition of the acetylated histone H4 tail. Many proteins require loop motion for the specific recognition of their substrate, cofactor, or protein partners in a closed form [35], therefore we can speculate that in ATAD2A, a closed form that is able to accommodate the acetyl histone peptide exists.

BC loop flexibility also accompanies the peptide recognition. In the H4K5ac complex, binding of the histone is associated with a concomitant flip of the P1065 peptide bond and the rearrangements of 2 residues (D1066 and R1075) which stabilize the BC loop in a peptide-compatible conformation (Fig. 2c).

Evidence of conformational flexibility of ATAD2A

Because we captured ATAD2A-H4K5ac complex in a closed form, we next investigated whether ATAD2A could also adopt multiple conformations in the absence of peptide. Such conformations could offer attractive opportunities for structure-guided drug design intervention, and paradoxically the flexibility may explain the weak affinity of ATAD2A ligands, and thus the recent experimental observation that ATAD2A is challenging to drug [20, 21]. Thus, we screened for new ATAD2A crystallography conditions, and obtained crystals in a number of different conditions. Amongst them, one crystal provided us with four novel peptide-free structures of apo-ATAD2A. Comparison of the reported apo

structure [24] with our four additional apo forms and the H4K5ac-bound complex provide an opportunity to understand the implications of dynamic flexibility in the ATAD2A bromodomain.

Superposition of the four additional apo forms revealed differences in ZA loop positions (Fig. 3a). A visual graphical scheme represented in Figure 3b highlights the relative movements of the ZA loop and describes 6 different states caught by X-ray crystallography. To compare the states, we used as a reference the position of the ZA loop observed in the ligand-free structure PDB3DAI, which is defined as state ①. Of the four additional apo forms, three of them are wider (states ②, ③ and ④) than the reference, and one form adopts an intermediate closed conformation (state ⑤), which was found in the H4K5ac-bound form (state ⑥). Overlay of the X-ray crystal structures of ATAD2A bound to fragments recently published [20, 21] showed that ligands bind in state ①. Surprisingly in one of the open states (state ④), our density map showed an unexpected dual conformation for the conserved asparagine (Supplementary Fig. 2b). In the predominant orientation, N1064 adopts a position commonly found in the bromodomain family by pointing within the acetyllysine-binding cavity (occupancy of 60%). Strikingly, in its alternate conformation, N1064 faces the BC loop and engages a hydrogen bond with the peptide-flipped carbonyl of P1065, assisting the transition into the histone-compatible conformation (Fig. 3c). The reversal of N1064 is supported by 2 additional hydrogen bonds involving residues from helix α C (D1071 and R1075). Thus, by assisting the peptide-bond flip of P1065, N1064 triggers the BC loop conformational change from the apo form to a histone-compatible state. Therefore, we can hypothesize that we caught a pre-activated state which sheds light onto an undescribed structural and functional role of the evolutionary conserved asparagine in ATAD2A.

H3K14ac binds to the C-helix of ATAD2A, a potential interface for protein-protein interaction

In the absence of a response from H3K14ac in our AlphaScreen assay, we attempted to co-crystallize ATAD2A in the presence of this histone peptide to check for specificity. Novel crystallography conditions were found. Surprisingly, the H3K14ac-bound complex solved at 2.7 Å reveals that H3K14ac interacts along the end of α C helix, far from the acetyllysine binding site (Figure 4a). The binding pose showed that the acetyllysine hydrogen bonds with 2 residues (R1005, H1076) and highlights the presence of a groove which accommodates the peptide backbone between the α Z and α C helices (Figure 4b). However, since bromodomains are protein-protein partner modules, we questioned whether this interaction mapped a previously unrecognized protein-protein interface. We performed computational simulations using Sitemap [36] and identified a potential protein-protein interface at the surface of ATAD2A which partially overlaps the experimentally observed H3K14ac binding pose (Figure 4c). Confirmation of the binding of H3K14ac at a site distal from the expected binding site raises the possibility of an additional binding surface which might have important implications in bromodomain biology.

Isoxazole-based small molecules used as ATAD2A chemical probes targeting the acetyllysine pocket

There were no reported ligands for ATAD2A when we initiated our studies, so we sought a suitable ligand with which to validate an ATAD2A AlphaScreen assay as well as to initiate a medicinal chemistry effort. We performed an X-ray crystallography screen with fragments as well as known acetyl-lysine mimetic templates. We identified two dimethylisoxazole-containing, compounds **1** and **2** (Fig. 5a, and Supplementary Fig. 3), which is a template that are known inhibitors of the well-studied bromodomains of the BET family [37-40]. We liked the modular features that this template provided and envisioned that an appropriately bi-substituted version would allow one to access both the RVF-shelf and the ZA-channel. We also tested the known broad spectrum bromodomain inhibitor Bromosporine (Fig 5a), referenced by the Structural Genomic Consortium website (<http://www.thesgc.org/chemical-probes/bromosporine>) in our AlphaScreen assay. We confirmed that the three molecules bound to the ATAD2A acetyllysine pocket (Supplementary Note 3, Supplementary Fig. 3c) with high μM affinities observed in both the AlphaScreen and an orthogonal DiscoverX competitive displacement assay (see Material and Methods) (Fig. 5a). This is in contrast with the low μM affinities reported for similar dimethylisoxazoles against the BET bromodomain family and corresponding ligand efficiencies of up to 0.39 [37-40]. Despite the weak affinities observed, compound **1** displayed a higher ligand efficiency of 0.28 than compound **2** and bromosporine (0.20 and 0.21, respectively) and provides a reasonable starting point from which to design more potent inhibitors. The modest ligand-efficiencies observed are similar to other recently reported fragments [20, 21] and likely reflect the lower druggability of ATAD2A versus other bromodomains such as BRD4. However, since structure-guided analysis revealed that the H4K5ac complex adopts a closed form, we investigated whether ATAD2A could adopt this state in presence of compound **1** and compound **2**.

The co-crystallization of compound **1** and compound **2** provided complexes in an ATAD2A open form (resolution of 1.90 Å and 1.74 Å, respectively) similar to the previously reported structure [20, 21, 24]. All structures superimposed well, showing no significant rearrangements of the ZA and BC loops, and the **2** complexes revealed an acetyllysine mimetic binding mode for both ligands (Supplementary Fig. 3c). These results validate the strategy we employed to develop a robust AlphaScreen assay and provided further insight as to the structural requirements for ligand versus peptide binding.

The binding pose of the dimethylisoxazolein compound **1** and compound **2** complexes revealed a common signature of key interactions, also reported in BET family [37-40] (Fig. 5b, c). In the ATAD2A bromodomain, the isoxazole moiety forms hydrogen bonds with N1064 and a conserved water molecule bridges the heterocycle to the conserved tyrosine Y1021 (Fig. 5b, c). Therefore, compound **1** and compound **2** are validated acetyllysine mimetics. However, to enter at the bottom of the narrow cavity, both complexes show that the orientation of the isoxazole is guided by hydrophobic contact from the ZA loop (V1013) and the 5-methyl substituent of the isoxazole (Fig. 5b, c). Due to these constraints, side chains of V1008 rearrange to prevent steric clashes, as exemplified by the binding mode of compound **1** (Fig. 5d). It has been demonstrated that a conformational variability of the

protein target can impair ligand affinity [41], therefore our structure-guided analysis provides an explanation of the weak affinity measured for these ligands. In addition, it is worth emphasizing that in the H4K5ac peptide-bound form, compounds **1** and **2** cannot enter the narrow acetyllysine binding pocket which explains why the ligand-bound complexes were obtained in an open form. Despite a common signature, each molecule displays specific interactions. While compound **1** interacts with the RVF shelf (salt bridge with R1007) (Fig. 5b), compound **2** loses this salt bridge. Instead, compound **2** expands to the ZA channel, interacts with the flexible part of the ZA loop through hydrogen bonds (D1014) and hydrophobic contacts (R1007, K1011, P1012, E1017 and V1018) (Fig. 5c). Phenyl ring orientation of compound **1** makes adjusted hydrophobic contact with the RVF shelf. On the contrary, the equivalent phenyl ring in compound **2** is rotated away in order to accommodate where the sulfonamide derivation of the small molecule anchors (Supplementary Fig. 3c). Therefore, the change of binding mode observed by X-ray crystallography correlates with the weaker binding measured *in-vitro* for compound **2** versus compound **1** (Fig. 5a).

Discussion

In the search for small molecule inhibitors of the human ATAD2A-acetylated histone complex, we characterized the H4K5ac peptide as a specific, acetyllysine binding site partner and highlighted evidence of protein dynamics in the ATAD2A bromodomain. From a structure-guided point of view, our findings are important because they clearly demonstrate that protein flexibility can impair protein-ligand interactions [41]. To our knowledge, such flexibility has not been described by X-ray crystallography for any bromodomain. The proline residues which define the loop motion are conserved in the family (Supplementary Fig. 4) suggesting that this loop flexibility is not unique to ATAD2A. Sorting bromodomains based on their flexibility may complement the well-established structural classification of bromodomains [24] and could improve the specific classification based on the acetyllysine binding sites that emerged from a computational druggability analyses [42].

Molecular dynamics simulations conducted by Steiner et al. predicted that the conserved asparagine in ATAD2A can adopt an alternate conformation [34]. Nevertheless, the movement of N1064 we observed experimentally is completely novel. In the simulation, the N1064 side chain points only within the active site, while we demonstrated that the ATAD2A flexibility is much larger than predicted, and N1064 can reverse its position to point between α B and α C helix. Based on our X-ray crystallographic snapshot, we propose that N1064 assists the BC loop transition into a histone compatible conformation. However, the role of the conserved asparagine may not be generalized to the entire bromodomain family because some histone peptides do not interact with the BC loop. Taken together our data support the results found in the molecular dynamics simulation and alternate conformations of N1064 have to be taken into account for future virtual screening campaigns.

From a rational design point of view, the predicted second binding site is not ideal for drug binding (Dscore 0.68) [42]. However, our study demonstrates that ATAD2A is flexible, thus the second binding site might become more accommodating if the hydrophobic residues rearrange to create a more enclosed site. This has been shown for some therapeutic targets,

as exemplified by the Bcl-2 family with ABT-737 inhibitor class [43]. Although speculative, the H3K14ac complex combined with the computational predictions suggest a potential site for protein or small molecule. Searching for chemical tools that could dock in an induced-fit second binding site might be envisioned to explore whether bromodomain rearrangement may provide a closed form allowing H4K5ac recognition.

Finally, by comparing the binding conformation of compounds 1 and 2 as well as other recently reported ligands rearrangement of V1018 may have an impact on ATAD2A ligand recognition. The flexibility of the ATAD2A bromodomain should be taken into account for further design of more potent ATAD2A inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations footnote

ATAD2A	ATPase family AAA domain containing 2 isoform A
EZH2	Enhancer of zeste homolog 2
DOT1L	Disruptor of telomere silencing 1-like
BET	Bromodomain and extra-terminal
BRD4	Bromodomain-containing protein 4
TCGA	The Cancer Genome Atlas
EF2	Elongation factor 2
ERα	estrogen receptor α
AR	androgen receptor
VCP/p97	Valosin-Containing Protein
Bcl-2 family	B-cell lymphoma 2 family
H4K5ac	H4K5 acetylated peptide
H3K14ac	H3K14 acetylated peptide
ITC	Isothermal Titration Calorimetry

Summary Statement

We demonstrate that ATAD2A bromodomain possess a conformational flexibility which participates in the recognition of the H4K5 acetylated histone peptide, and we identified acetyllysine mimetic inhibitors which revealed a more “open” conformation of the bromodomain binding-site of ATAD2A.

Figure 1. Structural basis for H4K5ac readout by ATAD2A

(a) General topology of ATAD2A in its apo form [21] (PDB3DAI). The ZA loop (T1003-D1030; Orange) possesses a bromodomain specific 3(10)-helix which forms a hydrophobic groove with specific RVF residues (the RVF shelf; 1007-RVF-1009). In addition, the ZA loop holds a short $\alpha Z'$ helix flanked by 3 prolines (P1012, P1019 and P1028; Red). Residues located before $\alpha Z'$ create a second groove (ZA channel) filled with conserved water molecules [35] and targeted for ligand optimization [44]. The BC loop is composed by 4 residues 1065-PDRD-1068, where the proline (P1065; Red) follows in the sequence the evolutionary conserved asparagine (N1064; Green) known to be essential for the histone recognition [21]. (b) Representation of the hydrophobic contacts found in H4K5ac complex: Hydrophobic contacts involve residues V1008 (3.8 Å), F1009 (4.1 Å), V1013 (3.8 Å) and I1074 (3.9 Å) at the bottom of the acetyllysine binding pocket, and V1018 (two contacts of 4.1 Å and 3.8 Å) and Y1063 (3.9 Å) at the upper part of the acetyllysine binding pocket. (c) Representation of the hydrogen bonds found in H4K5ac complex: Acetyllysine side chain interacts directly with N1064 (3.5 Å) and indirectly with Y1021 via one water molecule (Carboxy-Water1-Y1021, with hydrogen bonds of 2.5 Å and 2.7 Å respectively) and with V1008 via 2 water molecules (Ne-Water1-Water2-V1008, with hydrogen bonds of 2.8 Å, 2.9 Å and 2.5 Å respectively). Acetyllysine backbone is also forming an additional hydrogen bond with N1064 through a water molecule. R3 side chain of H4K5ac peptide (R3, blue) interacts firmly with 2 carbonyls from the backbone of E1062 and Y1063 at the end of the αB helix (3.2 and 3.1 Å, respectively).

Figure 2. ATAD2A loop motion accompanies histone recognition

(a) Front view of the superposition (along α C helix) showing flexibility of ZA and BC loops. (b) Front view highlighting the ZA loop motion mediated by P1012 and P1028. Note the C_{α} shifts of V1013 and V1018 by 1.1 Å and 1.7 Å, respectively. (c) Top and back views represent the BC loop accommodation upon histone binding. In the empty structure P1065 and D1066 are maintained by R1075. A water molecule present only in the apo form is shown as a small sphere. In the H4K5ac peptide bond structure, R1075 rearranges to create a new hydrogen bond network and interacts with L1061 holding P1065 in its new conformation. In the back view, atoms from R3 are represented as spheres to illustrate the steric clash encountered between the peptide residue and the P1065 position seen in the apo form. For all panels, loops from the apo form (PDB3DAI) and the H4K5ac-bound complex are represented in yellow and green, respectively.

Figure 3. Conformal flexibility of ATAD2A

(a) Representation of various ATAD2A states caught by X-ray crystallography highlighting flexibility properties of the ZA loop. Red, yellow and green ZA loop color-code ranks the ATAD2A states from a wide open to a narrow closed states, respectively. To locate the acetyllysine binding site, V1018 side chain and H4K5ac peptide are represented. (b) Graphical scheme of the relative movement of the ZA loop and states classification. Position of the ZA loop seen in the SGC apo structure (PDB ID 3DAI) has been used as a state ①. Quantification of the relative movement is described in Material and Methods. (c) Pre-activated state and dual conformation of the evolutionary conserved N1064 (Green) revealed by X-ray crystallography. In its alternate conformation, N1064 interacts with D1071 and R1075 (Yellow) and maintains the carbonyl of the P1065 which has flipped (Orange).

Figure 4. H3K14ac binds non-specifically to ATAD2A

(a) Surface representation of H4K5ac complex (left panel, peptide in yellow) and nonspecific binding of H3K14ac along α C (right panel, peptide in red) observed by X-ray crystallography. For all panels, in order to locate the acetyllysine binding pocket, the side chain of N1064 is shown in green. (b) ATAD2A groove (yellow surface) revealed by H3K14ac complex. Residues forming hydrogen bonds with the acetyllysine (R1005 and H1076) are represented in blue. (c) Computational predictions of a potential site for small molecule at the surface of ATAD2A overlap partially with the H3K14ac binding pose observed by X-ray. The residues involved in the predicted cavity are as follows: Hydrophilic residues: R999, R1005, T1084, E1091, E1092. Hydrophobic Residues: Aliphatic side chain of R999, F1006, T1084 (methyl) and I1088.

Figure 5. Structure-guided analysis of compound 1 and compound 2 in complex with ATAD2A
 (a) Chemical structures of bromosporin, compound 1, compound 2 and their reported IC_{50} , K_d , and Ligand Efficiency (LE). (b) and (c) Schematic representations of the molecular interactions with compound 1 and compound 2, respectively. Two dimensional schemes were generated using Ligplot+ [45]. For both complexes, hydrophobic contact from V1013 is highlighted in yellow, contacts with V1008 is shown in purple. Hydrogen bonds are represented with dashed red lines. (d) ATAD2A representation in sphere in a ligand free (left) and compound 1 bound form (right). Compound 1 is represented in green, V1013 in yellow; V1008 which rearranges to accommodate the ligand is shown in purple.