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Synthesis, Biochemical Characterization, and Genetic Encoding of a 1,2,4-Triazole Amino Acid as an Acetyllysine Mimic for Bromodomains of the BET Family

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Abstract: Lysine acetylation is a charge-neutralizing post-translational modification of proteins bound by bromodomains (Brds). A 1,2,4-triazole amino acid (ApmTri) was established as acetyllysine (Kac) mimic recruiting Brds of the BET family in contrast to glutamine commonly used for simulating this modification. Optimization of triazole substituents and side chain spacing allowed BET Brd recruitment to ApmTri-containing peptides with affinities similar to native substrates. Crystal structures of ApmTri-containing peptides in complex with two BET Brds revealed the binding mode which mirrored that of Kac ligands. ApmTri was genetically encoded and recombinant ApmTri-containing proteins co-enriched BRD3(2) from cellular lysates. This interaction was blocked by BET inhibitor JQ1. With genetically encoded ApmTri, biochemistry is now provided with a stable Kac mimic reflecting charge neutralization and Brd recruitment, allowing new investigations into BET proteins in vitro and in vivo.

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Introduction

Post-translational modifications (PTMs) are important means of regulating protein function and activity.[1] Lysine acetylation is a highly abundant PTM that neutralizes the charge at the side chain und constitutes binding sites for acetyllysine (Kac) reader-modules.[2] Lysine acetylation was initially discovered on histone proteins packaging eukaryotic DNA into chromatin. The charge-neutralizing effect of lysine acetylation impacts chromatin condensation and accessibility.[3] This PTM further interacts with Kac binding modules referred to as bromodomains (Brds), which are imbedded into several chromatin factors facilitating their recruitment to the DNA template (Figure 1a).[4] Lysine acetylation is installed and erased by lysine acetyltransferases (KATs) and lysine deacetylases (KDACs), which jointly control acetylation levels of histone and non-histone proteins.[5] The abundance and dynamic nature of this PTM complicates investigations into its complex biology and synthetic lysine analogs have supported research into this modification.[6] Biochemical research relies on stable mimics of the acetylated and deacetylated states, which cannot be altered by KAT or KDAC activities.[7] The most commonly used Kac mimic is glutamine, which is introduced into proteins by site-directed mutagenesis.[8] While glutamine simulates the charge-altering effect of lysine acetylation, it does not bind Brds. This shortcoming also applies to mimics of protein phosphorylation, where the modified serine residue is commonly mutated to glutamic acid. However, chemical biology provides artificial amino acids, such as phosphonomethylene alanine (Pma), which recapitulate charge alteration and recruitment of phospho-serine binding modules.[9] Despite the importance of lysine acetylation, only a few examples of stable non-natural amino acids have been reported as Kac mimics. These include Nε-methanesulfonyl-lysine and methylthiocarbonyl-thia-lysine, which show limited interactions with selected Brds.[10] Recently, Conway and co-workers reported a set of isoxazole-containing amino acids that can displace lysine acetylated peptides from Brds and are able to substitute for Kac at selected positions in a poly-acetylated peptide.[11] These reports demonstrate the general feasibility of employing non-natural amino acids as Kac mimics and encourage the development of new stable Kac mimics for applications in biochemical and biological research. Important features of such Kac mimics include the ability to bind Brds with similar affinity

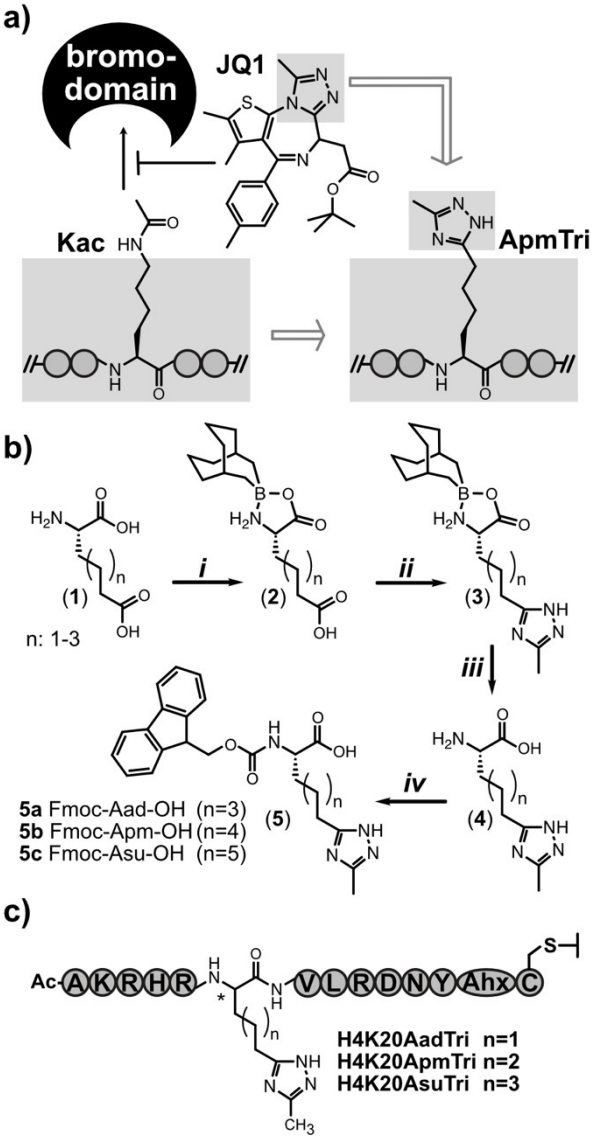

Figure 1. Triazole amino acids as mimics of acetyllysine. a) Brds bind acetylated lysine residues imbedded into sequence contexts. These interactions are blocked by triazole-containing small molecule ligand JQ1. Acetyllysine mimic ApmTri was generated by grafting a 1,2,4-triazole moiety into an amino acid scaffold. b) Synthesis Scheme of triazole amino acid building blocks for solid-phase peptide synthesis. *i*: 9-BBN, THF/MeOH, reflux; *ii*: acetamidine, COMU, DMF, 2 h 50 °C, followed by hydrazine, acetic acid, 2 h, 80 °C; *iii*: TFA, 1 h, 50 °C; *iv*: Fmoc-OSu, $NaHCO_3$, H_2O/1,4-dioxane, 2 h, RT. c) Immobilized H4K20 probes with triazole amino acids substituting K20.

as native Kac, a binding mode understood at the molecular level and established incorporation into recombinant proteins by means of molecular biology.

Results and Discussion

Here we report a new Kac mimic for biological research, its biochemical investigation and genetic encoding. We focused on probes for Brds of the Bromodomain and Extra-Terminal Domain (BET) family, which are part of the chromatin factors BRD3 and BRD4.[12] These proteins possess two adjacent Brds recruiting BRD3 and BRD4 to the chromatin template. Both proteins have gained considerable attention as drug targets due to overexpression in various types of cancers and formation of oncogenic fusion proteins.[13] Small molecule ligands of BET Brds, such as JQ1 and iBET, have been developed to block BET protein recruitment to chromatin by competing for the Kac binding sites (Figure 1a).[4d,14] These so-called BET inhibitors served as basis for the development of the Kac mimicking amino acid reported here. A crucial interaction between BET Brds and Kac residues is a hydrogen bond between a conserved asparagine residue and the Nε amide oxygen anchoring Kac into the binding pocket.[15] Inspection of the crystal structure of the first Brd of BRD4 – BRD4(1) – with bound JQ1 (PDB-ID: 3MXF) showed a similar interaction mediated by the 1,2,4-triazole of JQ1.[14a] Other small molecule BET inhibitors contain 1,2,4-triazoles as well and triazoles are in general considered as non-classical bioisosteres of amide bonds.[16] We reasoned that grafting a 1,2,4-triazole onto an amino acid scaffold could result in a metabolically stable non-natural amino acid able of BET Brd binding.

In a first step we developed a synthesis strategy for 1,2,4-triazole-containing amino acids. The Einhorn-Brunner reaction provides a synthesis route to substituted 1,2,4-triazoles.[17] A more recent variant of this reaction allows simple access to a reactive acyl amidine intermediate from carboxylic acids and amidines that cyclizes into 1,2,4-triazoles in presence of hydrazine.[18] Taking these developments into account, our synthesis strategy was based on commercially available dicarboxylic amino acids **1** of various side chain lengths which were first protected by complexation with 9-borabicyclo[3.3.1]nonane (9-BBN) (Figure 1b, Supporting Figure S1).[19] Protected compounds **2** were coupled with acetamidine and subsequently cyclized into 1,2,4-triazoles **3** by treatment with hydrazine. Next, the 9-BBN was removed resulting in **4**, followed by installation of the Fmoc-protection group, furnishing triazole amino acid building blocks **5**. We synthesized the L-enantiomers of three triazole residues with 3 to 5 methylene groups as spacer (Figure 1b and Supporting Table S1). The synthesis of 2-aminopimelic acid-derived triazole (ApmTri) required an additional chiral resolution step since the starting material was not available in enantiopure form (Supporting Figure S1). In the following, the triazole amino acid building blocks were incorporated as Kac substitutes at position 20 of a histone H4 peptide by solid-phase peptide synthesis (Figure 1c). The H4K20ac sequence is a known binding site of the second Brd of BRD3 (BRD3(2)).[4a] The three triazole peptides and controls containing unmodified (H4K20), acetylated (H4K20ac) lysine and glutamine (H4K20Q) were immobilized and subjected to pull-down assays with recombinant BRD3(2) fused to TurboYFP (Supporting Figure S2 and Supporting Tables S2 and S3).[20] As expected, BRD3(2)-TurboYFP was efficiently recruited to H4K20ac and did not bind H4K20 or H4K20Q (Figure 2a). The triazole probe H4K20ApmTri (4-methylene spacer) retained

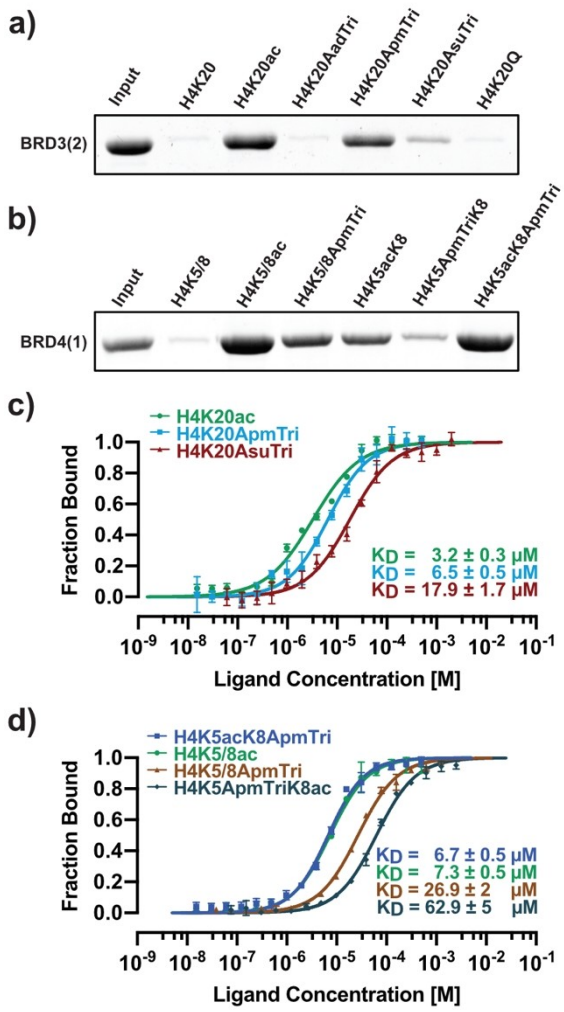

Figure 2. Binding assays with Brds and peptide substrates. a) Pull-down assays with BRD3(2)-TurboYFP and immobilized H4 substrates modified at the K20 site. The SDS-PAGE gel shows the protein fractions eluted off the probes after pull-downs with 10.5 µM BRD3(2)-TurboYFP. b) SDS-PAGE of pull-down experiments with 6.8 µM BRD4(1)-TagGFP2 and H4 probes modified at K5 and K8. c) Microscale thermophoresis (MST) assays with BRD3(2) and H4 substrates modified at the K20 site. The fraction of BRD3(2) in complex with the H4 peptide substrates was plotted against the ligand concentration in log scale and dissociation constants were extracted from the midpoints of the binding curves with the MO.Affinity Analysis v2.3 software package. d) MST assays with BRD4(1) and H4 ligands modified at K5 and K8.

BRD3(2)-TurboYFP efficiently, while H4K20AsuTri (5-methylene spacer) interacted only weakly and H4K20AadTri (3-methylene spacer) showed no interaction with BRD3(2)-TurboYFP (Figure 2a). These findings indicated that the 4-methylene spacer in ApmTri was well suited for positioning the triazole in the Kac binding pocket. To further confirm that ApmTri is recognized as Kac mimic and does not simply serve as covalent tether of the triazole, which binds BRD3(2) independently of the amino acid and peptide context, we synthesized a new set of probes for exploring the impact of ApmTri stereochemistry on Brd binding. H4K20ApmTri probes with enantiopure L-ApmTri and D-ApmTri, as well as racemic D/L-ApmTri were synthesized (Supporting Table S2). Pull-down experiments showed that the L-ApmTri probe recruited BRD3(2) in contrast to the D-ApmTri peptide (Supporting Figure S3a). The probe with racemic D/L-ApmTri showed reduced BRD3(2) recruitment which likely stems from the presence of the L-enantiomer in the racemate.

In the following, we investigated the impact of the C-3 methyl substituent of the triazole which is important for BET binding of small molecule ligands.[4e] Replacing acetamidine with formamidine, propionamidine or benzamidine during the synthesis resulted in three ApmTri derivatives that either lacked the substituent, or contained an ethyl or phenyl group at C-3 (Supporting Figure S1). However, none of the corresponding H4K20ApmTri peptides bound BRD3(2)-TurboYFP in pull-down assays, suggesting a crucial role of the C-3 methyl group in Brd binding, probably by reflecting the C-2 methyl moiety in the acetyl group of native Kac (Supporting Figure S3b).

Triazole-containing small molecule BET inhibitors like JQ1 and iBET are known to be specific for Brds of the BET family and do not bind non-BET Brds.[14a] The reason for this selectivity needs to be determined. In order to explore if this type of BET selectivity is also preserved in ApmTri, we probed further BET and non-BET Brds with triazole peptide substrates. The non-BET Brds of BAZ2B and CBP bind H3K14ac and H3K56ac, respectively and were probed with peptide probes containing AadTri, ApmTri, or AsuTri (Supporting Figure S3c). However, both non-BET Brds did not bind to any probes containing triazole amino acids. On the contrary, BET Brd BRD4(2) was efficiently recruited to H4K20ApmTri (Supporting Figure S4). We further observed that JQ1 could block BRD3(2)-recruitment to immobilized H4K20ApmTri and H4K20ac in contrast to non-BET inhibitor GSK2801 targeting the BAZ2B-Brd (Supporting Figure S5).[21] Collectively, these experiments showed that the binding preferences of JQ1 and related compounds are preserved in peptide probes containing ApmTri.

We continued investigating the first Brd of BRD4 fused to TagGFP2 (BRD4(1)-TagGFP2) (Supporting Figure S2). This BET Brd possesses an unusual binding mode by cooperatively binding two Kac residues in a single substrate peptide.[4b] In histone H4 acetylated lysines 5 and 8 are known to jointly bind BRD4(1). We synthesized a set of H4 peptide probes with ApmTri, Kac, and unmodified lysine at these sites (Supporting Table S2). Pull-down experiments with BRD4(1)-TagGFP2 showed recruitment to immobilized diacetylated H4K5/8ac and to a lesser extent binding of H4K5/8ApmTri containing ApmTri at both positions (Figure 2b). The control probe with unmodified lysines (H4K5/8) showed no recruitment of BRD4(1)-TagGFP2. Further probes aiming to investigate the impact of the individual sites showed that a lack of modification at K8 resulted in reduced BRD4(1)-TagGFP2 recruitment (Supporting Figure S6). A mixed probe with native Kac at position 5 and ApmTri at position 8 (H4K5acK8ApmTri) showed BRD4(1)-TagGFP2 recruitment to the same level as the diacetylated probe (Figure 2b). Collectively, these findings showed

that ApmTri also serves as Kac mimic for BRD4(1) and that this residue is most efficient when introduced at position 8. At the K5 site ApmTri recruited BRD4(1) weaker than native Kac, but H4 substrates with ApmTri at both sites bound BRD4(1) with only moderately reduced efficiency when compared to the diacetylated substrate.

In the following we measured dissociation constants of the BET Brds in peptide substrate complexes by microscale thermophoresis (MST). The recorded K_D values of BRD3(2) bound to H4K20ac and H4K20ApmTri were 3.2 ± 0.3 μM and 6.5 ± 0.5 μM, respectively (Figure 2c). The interaction between BRD3(2) and H4K20AsuTri was 5.6-fold weaker (17.9 ± 1.7 μM) when compared to the native substrate (Figure 2c). MST assays of BRD4(1) with H4K5/8ac and H4K5acK8ApmTri showed K_D values of 7.3 ± 0.5 μM and 6.7 ± 0.5 μM, while the affinity of BRD3(2) for H4K5/8ApmTri (K_D: 26.9 ± 2 μM) was 3.7-fold weaker when compared to diacetylated H4 peptide (Figure 2d). An 8.3-fold higher K_D (62.9 ± 5.0 μM) was measured for the dissociation of the BRD4(1)-H4K5ApmTriK8ac complex. H4 substrates with a single Kac or ApmTri residue at position 5 or 8 showed strongly reduced BRD4(1) binding (Supporting Figure S6). The MST assays were in agreement with the results of the pull-down assays and further showed a strong reduction in binding affinity if only one of the lysine residues contained Kac or ApmTri. Importantly, the measured affinities for ApmTri substrates were similar or only moderately reduced when compared to Kac peptides.

Next, we investigated the interaction between ApmTri-containing peptides and BET Brds on molecular level by solving crystal structures of BRD3(2) in complex with H4K20ApmTri (PDB-ID: 8B5A) and BRD4(1) bound to H4K5acK8ApmTri (PDB-ID: 8B5B) and H4K5/8ApmTri (PDB-ID: 8B5C).[22] Both Brd constructs were expressed and purified without fluorescent tags, followed by crystallization with the respective peptide substrates. The structure of BRD3(2) at a resolution of 1.92 Å displays the conserved Brd fold with four helices connected by two flexible loops (Supporting Figure S7a). The peptide substrate is well defined by electron density, clearly showing that ApmTri inserts into the Kac binding pocket. Nitrogen atoms N-1 and N-2 of the triazole approach N391 of BRD3(2), enabling hydrogen bonding reflecting the anchoring interaction of native substrates (Figure 3a). In addition, the triazole is in contact with Y348 via a water molecule, reminiscent of a similar interaction between Y348 and the Nε amide in Kac-containing peptides.[23] When comparing the binding mode of H4K20ApmTri to that of JQ1 and a H3K18ac peptide ligand by alignment of the corresponding BRD3(2) structures, we observed superpositioning of the triazoles and the acetyl group, further supporting the hypothesis that the triazole moiety in ApmTri serves as non-classical bioisostere of the Nε amide in Kac (Supporting Figure S7c and S7d).

The cooperative binding of two Kac marks by a single BET Brd has been analyzed on molecular level for BRD4(1) and other BET Brds.[4b,24] The H4K5ac residue inserts into the Brd binding pocket and establishes an anchoring hydrogen bond with N140. The second Kac residue (H4K8ac) adopts a diagonal orientation and approaches H4K5ac along the surface. A bridging water molecule connects the Nε amides of both acetyl groups by hydrogen bonding. The

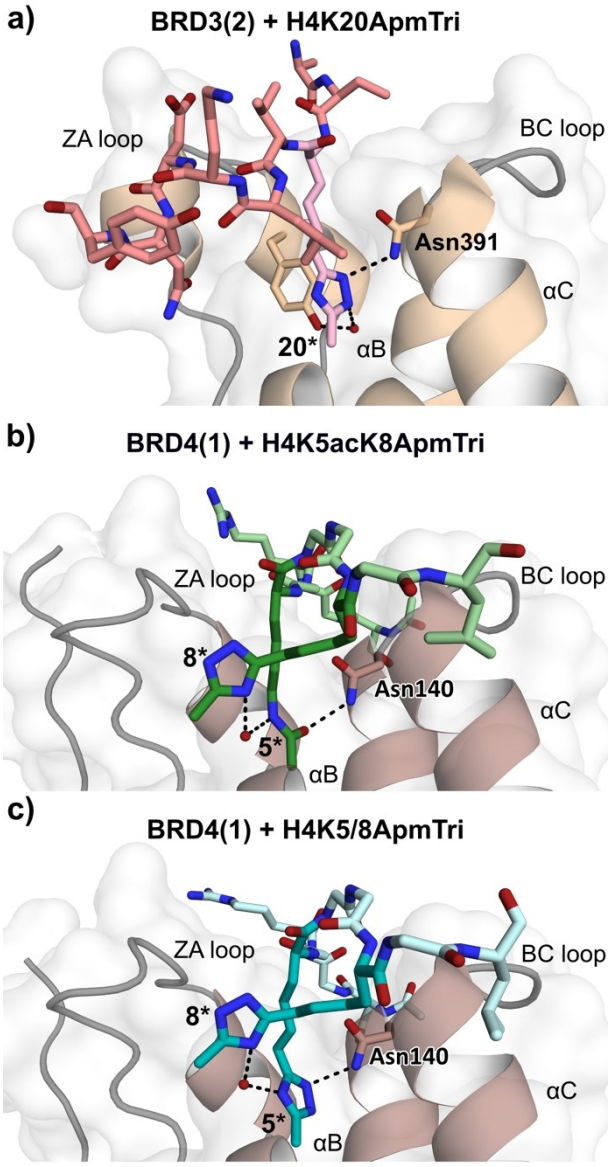

Figure 3. Crystal structures of BRD3(2) and BRD4(1) in complex with ApmTri-containing peptide substrates. Close-up view of the acetyllysine binding pocket of a) BRD3(2) (light brown) and b)-c) BRD4(1) (brown) bound to histone tail peptides containing acetyllysine mimic ApmTri. The ApmTri moiety forms a hydrogen bond with the evolutionary conserved asparagine (Asn391 in BRD3(2) and Asn140 in BRD4(1)). a) H4K20ApmTri (salmon) and interacting residues Asn391 and Tyr348 (light brown) are shown as sticks. b)-c) The ApmTri in position 8 sits on top of the acetyllysine binding pocket and is connected with the residue in position 5 via a bridging water molecule. H4K5acK8ApmTri (pale green) and H4K5/8ApmTri (pale cyan) interacting with residue Asn140 (light brown) of BRD4(1) are represented as sticks. The position of the ApmTri or acetylated lysine is highlighted in a) light pink, b) dark green or c) dark cyan. a)-c) The hydrogen bonds important for the acetyllysine or ApmTri binding are represented as black dashed lines. ApmTri or Kac residues in positions 5, 8 and 20 of the ligand peptides are indicated as 5*, 8* and 20*, respectively. PDB-IDs of a), b), and c) are 8B5A, 8B5B, and 8B5C, respectively.

crystal structures of BRD4(1) with bound H4K5acK8ApmTri and H4K5/8ApmTri, at 1.92 Å and 1.58 Å resolution, respectively, displayed the general Brd fold with the peptides in contact with the BC and ZA loops (Supporting Figure S8a). The H4K5acK8ApmTri structure showed the Kac residue at position 5 inserted into the binding pocket with the carbonyl hydrogen-bonded to N140 (Figure 3b). The amide nitrogen established a hydrogen bond with a water molecule connected to N-2 of the triazole of ApmTri in position 8. The diagonal orientation of H4K8ApmTri enabled this interaction with the bridging water. The structure of BRD4(1) in complex with H4K5/8ApmTri showed ApmTri in position 5 inserted into the binding pocket with triazole N-1 establishing the hydrogen bond with N140 and N-4 interacting with the bridging water molecule (Figure 3c). When comparing peptide ligand binding in both structures, the overlap of amide and triazole in position 5 was more pronounced than the overlap of the triazoles at position 8 (Supporting Figure S8b). The electron density of ApmTri at position 8 is less well defined in the H4K5/8ApmTri structure when compared to the structure with H4K5acK8ApmTri (Supporting Figure S8c and S8d). This might indicate a higher degree of conformational flexibility at position 8 of the H4K5/8ApmTri substrate. Alignments of BRD4(1) structures with bound JQ1 showed the overlapping triazoles at position 5 which served as primary Kac binding site (Supporting Figures S9a and S9b). A comparison with a BRD4(1) structure bound to a diacetylated H4 peptide further elucidated the similarity in residue confirmations at position 5 and variations in triazole orientation at position 8 (Supporting Figure S9c and S9d). Additional interactions between BRD4(1) and the ApmTri-containing peptide were observed (Supporting Table S4) and showed that the overall binding geometry of the ligands was preserved between ApmTri- and Kac-containing peptides.[4b] Collectively, the obtained crystal structures showed that ApmTri serves as Kac mimic with the triazole moiety establishing similar hydrogen bonding with the Brd-binding pocket as the native Kac residue.

In order to unlock the full potential of ApmTri as a Kac substitute and to enable biochemical and cell biological research with this non-canonical amino acid, we genetically encoded ApmTri. At first, we synthesized ApmTri without protection groups (Supporting Figure S1) and confirmed metabolic stability of ApmTri in cellular lysates (Supporting Figure S10). We resorted to the pyrrolysine system of *Methanosarcina mazei* for encoding ApmTri.[25] Based on the structure of ApmTri we tested if either of two PylRS variants accept this amino acid as a substrate. Specifically, we focused on the wild-type enzyme as well as an engineered PylRS variant that was designed for bulky lysine derivatives (Y306A, Y384F).[26] A GFP expression construct with amber codon in the coding sequence was used as reporter (Supporting Figure S2b). Successful Amber suppression in presence of ApmTri resulted in expression of full-length GFP indicated by increased fluorescence.[27] Gratifyingly, we observed efficient Amber suppression in HEK293 cells with the wild-type PylRS in presence of ApmTri (Supporting Figure S11). In the following, we cloned constructs to probe Brd binding with recombinant ApmTri proteins (Supporting Figure S2c). The H4K20 sequence including Amber codon at the K20 site was fused on genetic level to mCerulean3. The construct was further equipped with two affinity tags (StrepII-Tag and His6-Tag) for detection and enrichment from cell lysates. The DNA fragment was inserted into a bidirectional vector allowing co-expression with BRD3(2) fused to EYFP and the FLAG-epitope (Supporting Table S2). HEK293 cells transfected with the vector were treated with ApmTri or Nε Boc-protected lysine (KBoc) and correct expression of the constructs was confirmed (Supporting Figure S12a and S12b). KBoc is also incorporated into proteins during translation by PylRS/tRNA^{Pyl} in response to the Amber codon, but does not serve as Kac mimic (Supporting Figure S13). Afterwards, the cells were lysed and the H4-mCerulean3 proteins were precipitated in Strep-Tactin coated plates. Western Blot analysis confirmed expression of the full-length protein in presence of ApmTri and KBoc by immunostaining the C-terminal His6-Tag (Supporting Figure S12c). Immunostaining of the FLAG-tag further showed BRD3(2)-EYFP co-enriched with recombinant H4K20ApmTri, but not with the H4K20KBoc protein (Figure 4). Furthermore, JQ1 blocked BRD3(2) recruitment to the H4K20ApmTri protein (Figure 4), demonstrating the utility of genetically encoded ApmTri for investigating Brds und BET inhibitors.

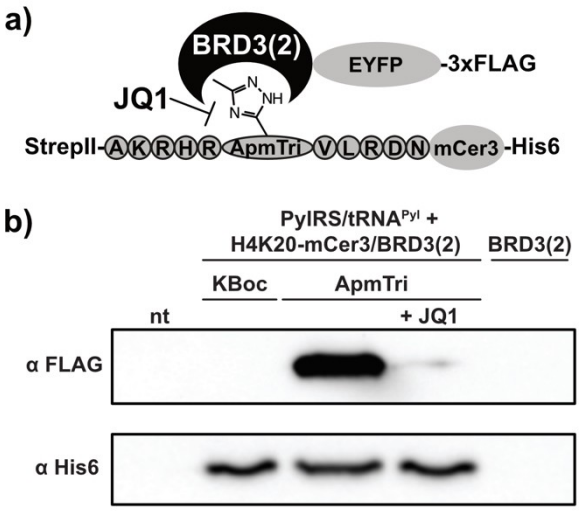

Figure 4. Incorporation of ApmTri into recombinant proteins with amber suppression technology. a) A BRD3(2) fusion protein with EYFP and C-terminal FLAG-tag was co-expressed to the H4K20 sequence fused to mCerulean3 (mCer3) and N-terminal StrepII- and C-terminal His6-tag. The inserted Amber codon in the H4 gene fragment allowed incorporation of ApmTri or KBoc by the wild type PylRS/tRNA^{Pyl} system. b) Upon expression in HEK293 cells the H4K20-mCer3 constructs are enriched from cell lysates on streptavidin. Western Blot analysis with antibodies against the His6-tag reports enrichment of recombinant H4K20-mCer3 proteins, while immunostaining with antibodies against the FLAG-tag reports co-precipitation of BRD3(2)-EYFP. Recruitment of BRD3(2) to H4K20ApmTri-mCer3 is blocked by excess of JQ1. nt: not treated.

Conclusion

In summary, we established the non-canonical amino acid ApmTri as a stable mimic of acetylated lysine residues for Brds of the BET family. In contrast to the commonly used Kac substitute glutamine, ApmTri reflects the charge-altering effect of Kac and is able to recruit Brds. The 1,2,4-triazole moiety serves as bioisostere of the Nε amide bond, which was confirmed by solving the structures of two BET Brds with bound ApmTri substrate peptides. ApmTri substrates interact with BET Brds with similar affinity as the native Kac residue, and genetically encoded ApmTri allows its incorporation into proteins in live cells. Since ApmTri cannot be erased by deacetylases, this new Kac mimic can enable new biochemical and cell biological investigations into the function and activity of BET Brds and BET inhibitors in vitro and in vivo. Furthermore, small molecule ligands carrying functional groups other than 1,2,4-triazoles for mimicking the acetyl moiety have been developed for BET and non-BET Brds.[14b,4e] Such inhibitors could be used for establishing a broad set of stable Kac mimicking amino acids in order to cover the complete set of mammalian Kac binding modules.

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Conflict of Interest

A patent was granted for ApmTri under file number: DE 102018214919 B4.

Keywords: Bromodomain · Chemical Biology · Genetic Code Expansion · Lysine Acetylation · Protein Modification

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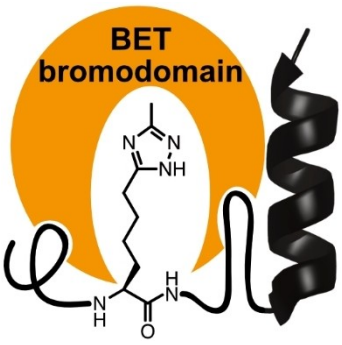
Research Articles

Chemical Biology

S. Kirchgäßner, M. B. Braun, N. Bartlick, C. Koç, C. D. Reinkemeier, E. A. Lemke, T. Stehle, D. Schwarzer* ____ **e202215460**

Synthesis, Biochemical Characterization, and Genetic Encoding of a 1,2,4-Triazole Amino Acid as an Acetyllysine Mimic for Bromodomains of the BET Family



A triazole-containing amino acid (ApmTri) was established as a mimic of acetyllysine for bromodomains of the BET family. Biochemical and structural investigations showed that ApmTri binds with similar affinity to bromodomains as acetyllysine and reflects the binding mode of the native modification at the atomic level. Genetic encoding enables ApmTri incorporation into proteins allowing investigations of bromodomain binding properties and inhibition.