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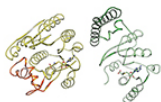
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## Bisubstrate analogues as structural tools to investigate m<sup>6</sup>A methyltransferase active sites

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### ABSTRACT

RNA methyltransferases (MTases) catalyse the transfer of a methyl group to their RNA substrates using most often S-adenosyl-L-methionine (SAM) as cofactor. Only few RNA-bound MTases structures are currently available due to the difficulties in crystallising RNA:protein complexes. The lack of complex structures results in poorly understood RNA recognition patterns and methylation reaction mechanisms. On the contrary, many cofactor-bound MTase structures are available, resulting in well-understood protein:cofactor recognition, that can guide the design of bisubstrate analogues that mimic the state at which both the substrate and the cofactor is bound. Such bisubstrate analogues were recently synthesized for proteins monomethylating the N6-atom of adenine (m<sup>6</sup>A). These proteins include, amongst others, RlmJ in *E. coli* and METLL3:METT14 and METTL16 in human. As a proof-of-concept, we here test the ability of the bisubstrate analogues to mimic the substrate:cofactor bound state during catalysis by studying their binding to RlmJ using differential scanning fluorimetry, isothermal titration calorimetry and X-ray crystallography. We find that the methylated adenine base binds in the correct pocket, and thus these analogues could potentially be used broadly to study the RNA recognition and catalytic mechanism of m<sup>6</sup>A MTases. Two bisubstrate analogues bind RlmJ with micro-molar affinity, and could serve as starting scaffolds for inhibitor design against m<sup>6</sup>A RNA MTases. The same analogues cause changes in the melting temperature of the m<sup>1</sup>A RNA MTase, TrmK, indicating non-selective protein:compound complex formation. Thus, optimization of these molecular scaffolds for m<sup>6</sup>A RNA MTase inhibition should aim to increase selectivity, as well as affinity.

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RNA binding; TrmK;  
RlmJ; m<sup>1</sup>A; bisubstrate  
analogues

### Introduction

The family of S-adenosyl methionine (SAM) dependent methyltransferases (MTases) comprises protein-, RNA- and DNA MTases. These proteins transfer methyl groups to different positions in protein side-chains, RNAs or DNAs, respectively. One subfamily of RNA MTases covers m<sup>6</sup>A RNA MTases that monomethylate the exocyclic N6-atom of adenine (m<sup>6</sup>A). This modification is found in all organisms from bacteria to human, and in both mRNA, tRNA, rRNA, small nucleolar RNA, non-coding RNA, and DNA [1,2]. m<sup>6</sup>A is involved in stabilizing RNA:RNA interactions as well as inducing RNA:protein interactions (recently reviewed in [3,4]).

A number of m<sup>6</sup>A RNA MTases have been structurally characterized as either full-length protein, e.g. Ribosomal RNA large subunit methyltransferase J (RlmJ) that methylates the 23S rRNA at position A2030 in *E. coli* [5,6], or as just the catalytic MTase domain as in e.g. eukaryotic methyltransferase like 16 protein (METTL16) that methylates pre-mRNAs, lncRNAs, and other ncRNAs [7–9], or METTL3 and METTL14 from the ternary complex of METTL3:METTL14 and the Wilms tumor 1 associating protein (WTAP) that introduces m<sup>6</sup>A into mRNAs


[10–12]. Of the known m<sup>6</sup>A RNA MTases, only METTL16 has been crystallised in complex with substrate RNA [13]. m<sup>6</sup>A RNA MTases are functionally and structurally related to m<sup>6</sup>A DNA MTases such as T4Dam or M.TaqI, both of which are structurally characterized in complex with substrate DNA (PDB: 1YFL, 1G38) [14,15].

In the absence of a structure of *E. coli* RlmJ in complex with substrate RNA, biochemical studies were performed to investigate substrate binding. These studies showed that RlmJ can methylate A2030 in a hairpin fragment of 23S rRNA as short as 25 bases, with the same efficiency as for full-length substrate [6]. The m<sup>6</sup>A2030 modification was suggested to enhance long-range stacking interactions within the rRNA, to increase the overall rRNA stability [2]. RlmJ methylates deproteinized 23S rRNA, indicating that the m<sup>6</sup>A2030 modification occurs early in the ribosome biogenesis [5]. The protein is none-essential, but has been associated with the ability of bacteria to use DNA as a nutrient [16], and with repression of plasmid uptake [17].

RNA MTases, as well as protein- and DNA MTases, have been linked to various types of cancer (reviewed in [18]) or

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rare mitochondrial respiratory chain complex deficiencies [19,20]. Of these, only protein- and DNA MTases have so far been targets for drug development. These drugs are either non-nucleosides or explore the scaffold of the methyl-donor cofactor SAM as a starting point for inhibitor design. SAM-derivate inhibitors include e.g. azacytidine (Vidaza, Celgene), decitabine (Dacogen, SuperGen), EPZ004777 [21], and DS-437 [22], the first two of which have been approved for clinical use. Currently, no inhibitors are known for RNA MTases other than the universal nucleoside analogue sinefungin and the natural cofactor product S-adenosyl-homocysteine (SAH).

Based on extensive structural comparison with other MTase families, and emerging evidence for links to human diseases, RNA MTases are increasingly considered promising drug targets [23]. Given the similarities in SAM cofactor binding pockets across MTase families, it was suggested to design drugs that simultaneously exploit the cofactor and substrate binding pockets [23]. Recently, a series of bisubstrate analogue encompassing an analogue of the cofactor SAM covalently linked to the N6-position of an adenosine, were synthesized [24]. These molecules aim to mimic the substrate:cofactor bound state in which a methyl group is transferred from SAM to the adenosine N6-atom of substrate RNA during catalysis by m<sup>6</sup>A RNA MTases.

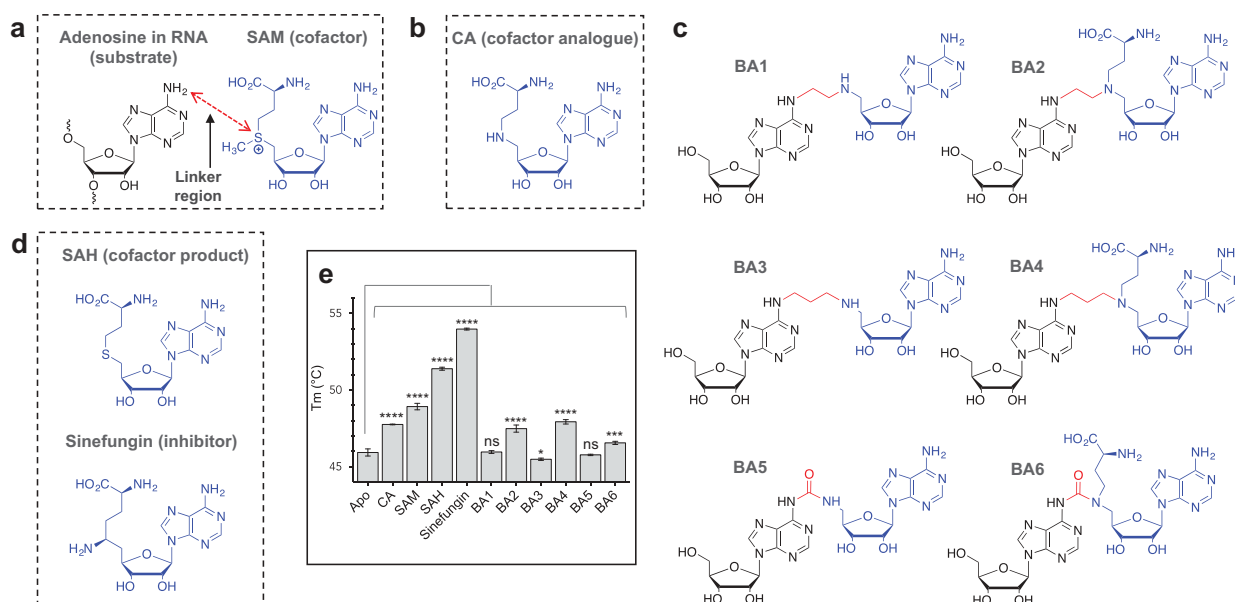
As a proof-of-concept, we use the m<sup>6</sup>A RNA MTase RlmJ as a model to test the binding mode of this series of bisubstrate analogues to assess their use as inhibitors against m<sup>6</sup>A RNA MTases, and their similarity in binding mode to the substrate:cofactor binding during methyl transfer. We further tested the broader capability of these bisubstrate analogues to bind to

an m<sup>1</sup>A RNA MTase protein (TrmK) related to RlmJ in structure.

## Results

### The bisubstrate analogues increase the T<sub>m</sub> of RlmJ

The bisubstrate analogues mimic the state at which the N6-atom of the substrate adenosine in RNA performs nucleophilic attack on the ε C-atom of the transferred methyl group in the natural cofactor SAM (Figure 1A). The synthesis of the bisubstrate analogues [24] entailed joining a cofactor analogue (CA) of SAM (Figure 1B) to the N6-atom of adenosine through a variety of linkers, yielding six molecules; bisubstrate analogue (BA) 1 to 6 (Figure 1C) [24]. To ensure diversity in the chemical space, the linker was synthesized as either a short chain of two C-atoms (Figure 1C, BA1 and -2), a longer chain of three C-atoms (Figure 1C, BA3 and -4), or a urea unit (Figure 1C, BA5 and -6). The bisubstrate analogues were further synthesized with (BA2, -4, -6) or without (BA1, -3, -5) the methionine group present in SAM and SAH. The binding of the CA and BA1-6, as well as the cofactor substrate SAM, cofactor product SAH, and the known inhibitor sinefungin (Figure 1D) to RlmJ, was evaluated using differential scanning fluorimetry (DSF). This technique measures the protein melting temperature (T<sub>m</sub>), and a change in protein T<sub>m</sub> in the presence of a bisubstrate analogue, implies formation of a protein:compound complex [25]. First, binding of the CA, SAM, SAH, and sinefungin were tested. RlmJ displayed an increased T<sub>m</sub>-value with all four molecules, compared to apo protein (Figure 1E). The four molecules vary only by the atom or chemical group at the δ-position: NH-group in CA, S<sup>+</sup>-CH<sub>3</sub> group in SAM,



**Figure 1.** Structure of the bisubstrate analogues and binding to RlmJ. (A) The structure of the substrate adenosine in RNA (black) and natural cofactor SAM (blue). The point of linkage in the bisubstrate analogues (BA) is indicated with a double arrow (red). (B) The structure of the cofactor analogue (CA) used as building block for the BA compounds. (C) A schematic overview of the structures of the six synthesized bisubstrate analogues; BA1 to 6. (D) The structure of the natural cofactor product SAH and universal RNA MTase inhibitor sinefungin. (E) The melting temperature (T<sub>m</sub>) for *E. coli* RlmJ as apo protein (no ligand) or in the presence of CA, SAM, SAH, sinefungin or one of six m<sup>6</sup>A RNA MTase bisubstrate analogues (BA1-6) from C, measured using differential scanning fluorimetry. P values are indicated as follows: ns = P > 0.05, \* = P ≤ 0.05, \*\*\*\* = P ≤ 0.0001, and \*\*\*\*\* = P ≤ 0.0001, n = 3.

S-atom in SAH, and C-NH<sub>2</sub> in sinefungin. The gradual increase in T<sub>m</sub> for RlmJ with CA < SAM < SAH < sinefungin indicates that the nature of the δ-atom is important for either direct interaction with the protein or for the ligand to adapt a suitable conformation. Next, the six bisubstrate analogues were tested for binding. These experiments yielded the largest T<sub>m</sub> shifts for RlmJ in the presence of the two compounds BA2 and BA4 (Figure 1E), indicating that these ligands form a larger number of stabilizing protein interactions, compared to BA1, -3, -5 and -6. A general T<sub>m</sub> comparison of all compounds showed that the methionine part likely is involved in protein binding (BA1, -3, -5 compared to BA2, -4, -6) and that an aliphatic linker is preferred (BA2, -4 compared to BA6) for optimal protein interaction with RlmJ.

### BA2 and BA4 are starting scaffolds for adenine-modifying MTase inhibitors

In recent years, MTases have received considerable attention as drug targets due to their link to human disease. The bisubstrate analogues, BA2 and BA4, yielded the largest shift in T<sub>m</sub> for RlmJ. To test the relevance of these analogues as inhibitors for RlmJ, K<sub>D</sub>-values were measured using isothermal titration calorimetry (ITC) for RlmJ in the presence of the SAM, sinefungin, BA2 or BA4. Sinefungin binds RlmJ with a K<sub>D</sub>-value of 1.7 ± 0.2 μM (Figure 2A) and SAH with a K<sub>D</sub> of 7.5 ± 0.9 μM (Figure 2B), highlighting the inhibitor potential of sinefungin. The ITC data revealed that the analogues, BA2 and BA4, bind RlmJ with low micro-molar affinity with K<sub>D</sub>-values of 25 ± 3 μM and 30 ± 4 μM, respectively (Figure 2C,D).

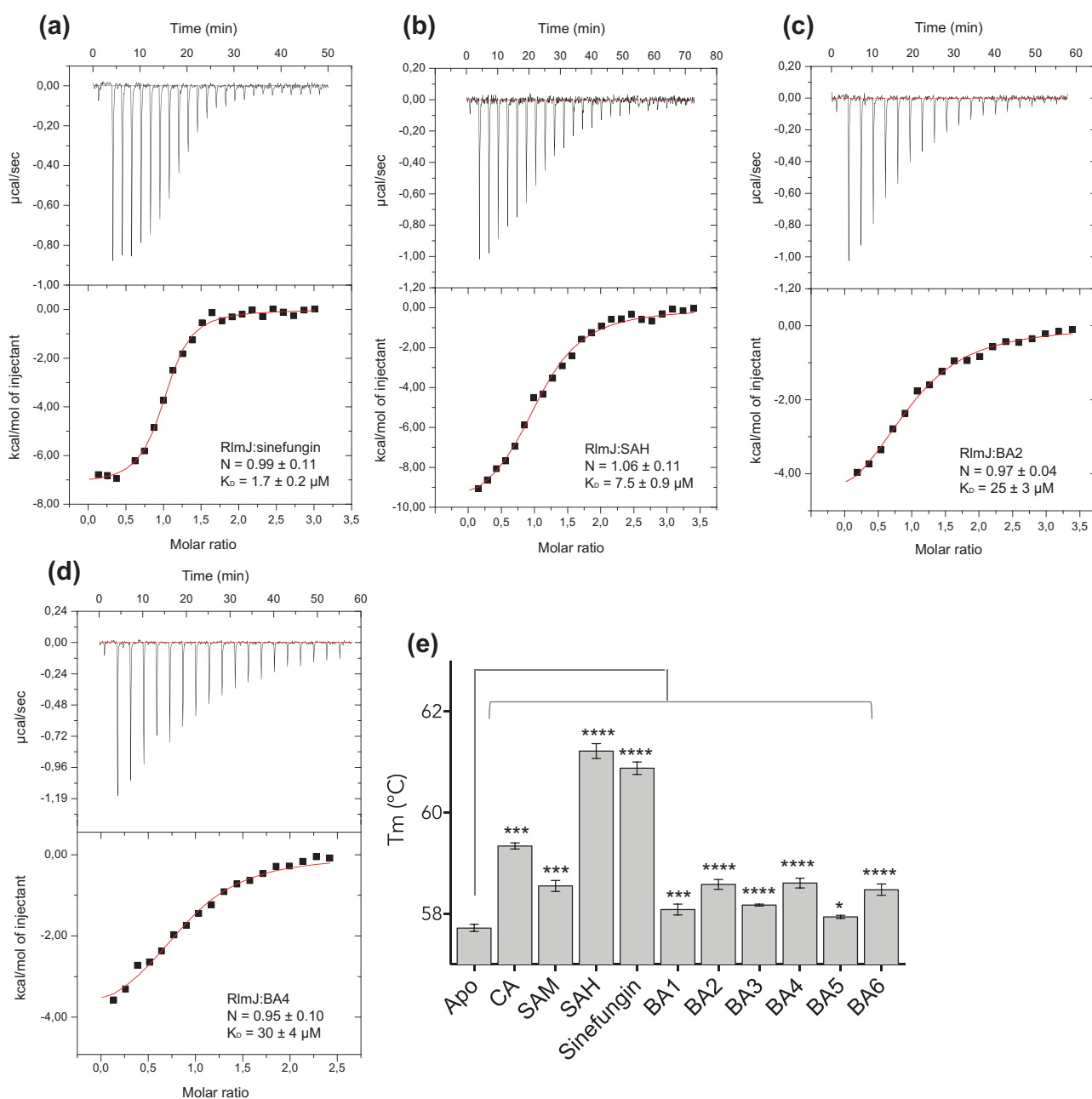
To test the specificity of the bisubstrate analogues against m<sup>6</sup>A RNA MTases, their binding to another adenine-modifying RNA MTase family was examined. The N1-monomethylation of adenine (m<sup>1</sup>A) has been extensively studied (reviewed in [26]), and thus we chose the m<sup>1</sup>A RNA MTase, TrmK, from *Mycoplasma capricolum*, that catalyzes formation of m<sup>1</sup>A in position 22 in tRNAs [27]. TrmK is essential for many pathogenic bacteria [28,29], and therefore serves as an appropriate target for inhibitor development. All six bisubstrate analogues, as well as CA, SAM, SAH and sinefungin were tested against TrmK using DSF as described. T<sub>m</sub>-values were increased in the presence of the CA, SAM, SAH, and sinefungin (Figure 2E). In contrast to the result for RlmJ, the CA here resulted in a higher ΔT<sub>m</sub> than SAM, suggesting that the N-atom in this molecule could be directly bound to residues from TrmK but not from RlmJ. Alternatively, binding of the CA could induce stabilizing conformational changes in TrmK, not occurring in RlmJ. For the bisubstrate analogues, BA2, BA4 and BA6, resulted in the largest T<sub>m</sub> increase for TrmK (Figure 2E). The binding of BA2 and BA4 increased the T<sub>m</sub> for both RlmJ and TrmK, suggesting that these analogues would not be inhibitory specific for m<sup>6</sup>A RNA MTases, but could instead be used as starting scaffolds for synthesis for both m<sup>1</sup>A and m<sup>6</sup>A RNA MTase inhibitors.

### Structure determination of RlmJ and TrmK bound to SAH, BA2, or BA4

To understand the T<sub>m</sub> and K<sub>D</sub> differences between bisubstrate analogues, the molecular binding mode of BA2 -or BA4 to protein was investigated. Crystallisation assays were performed with RlmJ or TrmK and each of these analogues. Experiments were also performed for each protein with SAH for later direct comparison with the cofactor part of BA2 and BA4. Crystals were obtained for RlmJ bound to SAH, BA2, or BA4. TrmK crystallised only in the presence of SAH. Structures were solved for RlmJ:SAH to 1.61 Å, RlmJ:BA2 to 1.39 Å, RlmJ:BA4 to 2.10 Å, and TrmK:SAH to 2.36 Å (Table 1), each with one ligand bound per active site. In RlmJ:BA4, the ligand adopted two conformations, where one occupied chain A and C, and one occupied chain B. The ligand in Chain D presented poor electron density and could only be partly fitted. The multiple binding modes for BA4 compared to the single mode for BA2 could explain the lower K<sub>D</sub>-value determined for BA4, compared to BA2. Interaction surface analysis [30] of the structures suggested, that both RlmJ and TrmK were monomers in solution, confirmed by size exclusion chromatography yielding a monomeric molecular weight (MW) for both proteins (Supplementary Figure 1A). The crystallised RlmJ structures display a class I MTase Rossmann-fold and a helical subdomain inserted at residues 47–98 (Figure 3A), similar to what was previously described [6]. The structure of TrmK:SAH displayed a class-I MTase Rossmann-fold core with an additional C-terminal extension (residues 179–245) (Figure 3B) comparable to TrmK from *Bacillus subtilis* (PDB: 6Q56). The core class-I Rossmann-fold of TrmK and RlmJ align with an RMSD of 2.9 Å<sup>2</sup> over 160 Ca. Such a similarity agrees with the DSF data suggesting that BA2 and BA4 could bind both proteins, and consistently, an alignment of TrmK:SAH with both RlmJ:BA2 and -BA4 shows that both ligands can be spatially accommodated in the active site of TrmK (Supplementary Figure S1B).

### The bisubstrate analogues bind in two conformations and near catalytic residues

In the RlmJ:BA4 structure, two distinctively different molecular conformations are observed for the BA4 molecule: a folded conformation, hereon named BA4(a) (Figure 4A,C), and an extended conformation, hereon named BA4(b) (Figure 4B,C). Each conformation was compared to the binding mode of SAH in RlmJ:SAH to analyze their applicability to mimic the substrate:cofactor binding state during methyl transfer. In RlmJ:SAH, the ligand binds in a pocket lined with hydrophobic residues and forms hydrogen bonds to residues H19, H42 and S100 via its methionine part, and E118, D143 and G144 via its adenosine part (Supplementary Figure S2A). Compared to SAH, most parts of BA4(a) binds out of the usual cofactor binding pocket (Figure 4A). π-π stacking of the two adenine moieties induces a folded conformation of the compound that displaces the central δ N-atom 1.7 Å from the expected position. The substrate part and the cofactor adenine of BA4(a) binds in between the cofactor and the expected substrate



**Figure 2.** Binding of bisubstrate analogues to RlmJ and TrmK. ITC curves for RlmJ with (A) SAH, (B) sinefungin, (C) BA2, and (D) BA4. Dissociation constant ( $K_D$ ) and stoichiometry ( $N$ ) values are reported as mean  $\pm$  standard deviation (SD). (E) The melting temperature ( $T_m$ ) of TrmK as apo (no ligand) or in the presence of CA, SAM, SAH, sinefungin or one of six m<sup>6</sup>A RNA MTase bisubstrate analogues (BA1-6), measured using differential scanning fluorimetry. P values are indicated as follows: \* =  $P \leq 0.05$ , \*\*\* =  $P \leq 0.001$ , and \*\*\*\* =  $P \leq 0.0001$ ,  $n = 3$ .

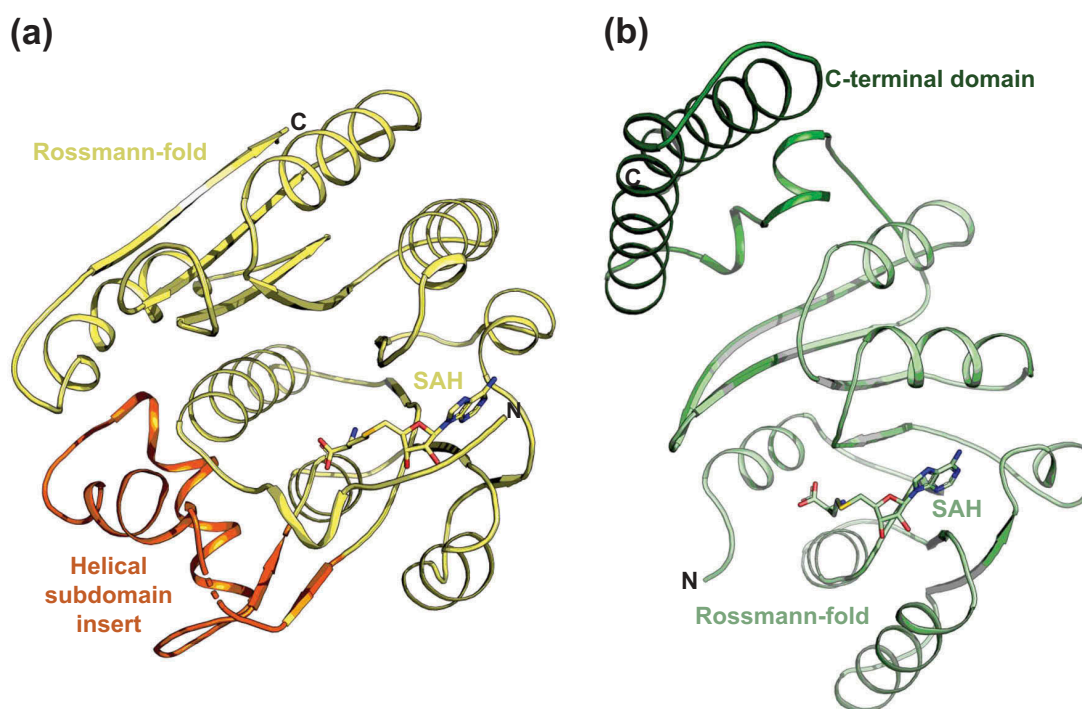
binding pocket, and as a result, BA4(a) shares only its interaction between the methionine part and residues H19 and H42 with SAH (Supplementary Figure S2C). A similar folded conformation was also assumed by BA2 in RlmJ:BA2 that, however, additionally shares the interaction with S100 with SAH (Supplementary Figure S2B,S3A,B). Contrary to BA4(a), BA4 (b) occupies both the presumed substrate binding pockets and parts of the cofactor binding pocket. In fact, relative to a possible real substrate:cofactor binding mode with RNA and SAM, BA4(b) deviates only by the orientation of the adenosine

in the cofactor part, which is rotated 120° out of the canonical binding pocket for SAH due to a shift in R/S-configuration around the central  $\delta$ -atom (Figure 4B). The substrate part of BA4(b) binds in the presumed substrate binding pocket, lined with hydrophobic and aromatic residues, and forms hydrogen bonds with residues N12, K18, and D164 (Supplementary Figure S2D). Two of these residues were shown to be catalytically important: the strictly conserved K18 residue, and the D164 residue from a conserved D/N-P-P-Y/F/W motif [6,15,31]. D164 forms a hydrogen bond of 3.0 Å to the N6-



Table 1. Crystallographic data. Processing and refinement statistics for the crystallised RimJ protein bound to SAH, BA2, or BA4, and TrmK bound to SAH.

	RimJ:SAH	RimJ:BA2	RimJ:BA4	TrmK:SAH
Data collection				
Beamline	ESRF ID23-2	Soleil PX2	Soleil PX2	ESRF ID23-2
Space group	P1	P 1 2 <sub>1</sub> 1	P 1 2 <sub>1</sub> 1	P 1 2 <sub>1</sub> 1
a, b, c	46.218, 46.224, 71.361	47.82, 58.40, 49.23	66.42, 170.73, 66.59	40.76, 131.09, 58.47
$\alpha$ , $\beta$ , $\gamma$	79.583, 77.595, 70.349	90, 97.32, 90	90, 118.32, 90	90, 103.88, 90
Molecule/ASU	0.87313	0.98010	0.98007	0.87313
Wavelength (Å)	43.23–1.61 (1.67–1.61)	37.46–1.39 (1.44–1.39)	48.24–2.10 (2.18–2.10)	42.91–2.36 (2.44–2.36)
Resolution range (Å)	236.903 (23.207)	340.809 (32.392)	428.800 (39.500)	167.982 (17.199)
Total reflections	67,854 (6668)	53,277 (5084)	75,173 (7404)	24,468 (2448)
Unique reflections	3.5 (3.5)	6.4 (6.4)	5.7 (5.3)	6.9 (7.0)
Multiplicity	97.11 (95.82)	98.68 (92.40)	99.00 (97.48)	99.75 (99.43)
Completeness (%)	9.89 (2.26)	12.71 (0.52)	5.63 (1.40)	5.47 (1.09)
I/ $\sigma$ (I)	0.997 (0.808)	0.999 (0.43)	0.98 (0.675)	0.996 (0.823)
CC <sub>1/2</sub>	0.07581 (0.4406)	0.0759 (2.033)	0.2072 (0.7769)	0.2719 (1.5)
R <sub>merge</sub> (%)				
Structure refinement				
R <sub>work</sub> (%)	0.1888 (0.2571)	0.2244 (0.4486)	0.2094 (0.3084)	0.1847 (0.2645)
R <sub>free</sub> (%)	0.2217 (0.3153)	0.2657 (0.4859)	0.2512 (0.3084)	0.2514 (0.3555)
RMSD <sub>bond</sub> (Å)	0.006	0.006	0.008	0.008
RMSD <sub>angle</sub> (°)	0.94	0.91	1.06	0.91
No. of non-hydrogen atoms	4735	2552	9598	3964
Macromolecules	4450	2237	8699	3776
Solvent	285	268	724	188
Ramachandran favored	96.81	97.45	96.84	98.21
Ramachandran outliers	0.00	0.00	0.00	0.00
Rotamer outliers (%)	0.00	0.00	0.00	0.00
Average B-factor (Å <sup>2</sup> ) of	17.11	30.19	29.36	41.06
Macromolecules	16.81	29.31	28.69	41.04
Solvent	21.88	37.66	35.14	41.46
PDB entry ID	6QES	6QEO	6QDX	6QEE



**Figure 3.** Crystal structures of RlmJ and TrmK. (A) RlmJ bound to SAH. The core Rossmann-fold is shown in yellow. The helical subdomain insert is shown in orange. SAH is shown in stick representation in yellow. (B) The crystal structure of TrmK bound to SAH. The core Rossmann-fold is shown in light green. The C-terminal domain is shown in dark green. The SAH ligand is shown in stick representation.

atom of the substrate base, and of 2.8 Å to the cofactor part of BA4(b) (Figure 4D). It is stabilized by a hydrogen bond to K18 (2.7 Å), that further binds to the N1-atom (3.2 Å) of the substrate base (Figure 4D). In this position, D164 could act as the suggested proton acceptor during catalysis by increasing the electron density of the N6-atom [6,15], and K18 is positioned such that it fixates D164 for catalysis. The N6-atom is positioned 2.5 Å away from the ε C-atom (Figure 4B). Such a distance, in two non-linked moieties, would allow for an  $S_N2$  methyl transfer to the N6-atom after its deprotonation [31].

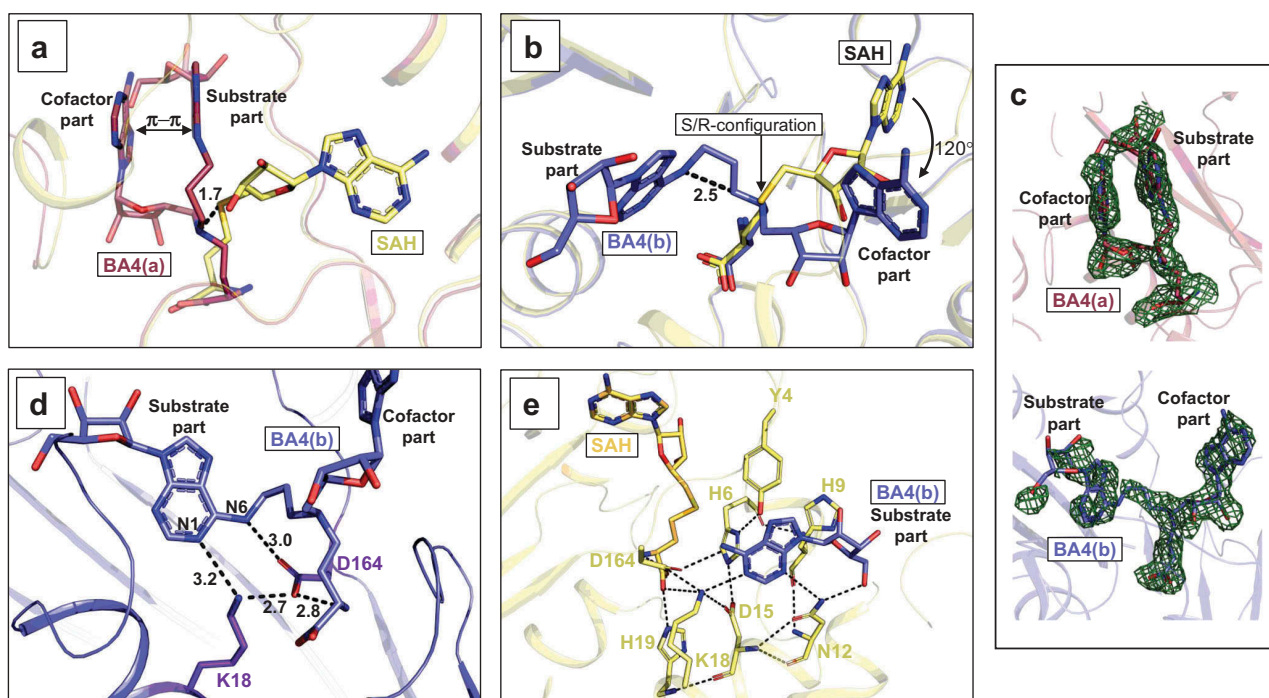
Y4 and H6 from the N-terminal region of RlmJ were also shown to be important for activity, and suggested to contribute to substrate binding [6]. The first 19 residues at the N-terminal are highly flexible and rotate up to 88° between the apo open conformation and the SAH-bound closed conformation [6]. A closed conformation of the N-terminal would sterically clash with parts of the BA molecules, and thus these ligand-bound structures assume the open conformation. For BA4(b), these clashes are solely with the rotated adenosine from the cofactor part, and thus the substrate base alone can be fitted nicely into the closed conformation of RlmJ:SAH (Figure 4E). In this fit, the two residues Y4 and H6 point towards the substrate base. The side-chain O-atom of Y4 was shown to be more important for activity than the aromatic ring [6]. In agreement with this, no  $\pi$ - $\pi$  stacking occurs between Y4 and the substrate base. Instead, this O-atom is part of an intricate network of hydrogen bonds that ultimately binds to the base via the catalytically important K18 (Y4-H6-D15-L18-base) (Figure 4E). The close proximity of the substrate base from BA4(b) to the catalytically important residues (D164 and K18) of RlmJ, implies, that the extended

conformation of BA4(b) resembles the real  $m^6A$  RNA MTase substrate:cofactor bound state.

#### **Ba4(b) is applicable for studying RNA recognition in all $m^6A$ RNA and DNA MTases**

Structural similarities of the active site across  $m^6A$  RNA MTase suggest that the substrate analogues, tested here for RlmJ, could also be used to investigate RNA recognition of other  $m^6A$  RNA MTases. The crystal structure of the  $m^6A$  RNA MTase METTL16 has been solved in complex with substrate RNA [13] without the cofactor, and the Rossmann-fold of RlmJ aligns with this protein (PDB: 6DU4) with an RMSD of 2.9 Å<sup>2</sup> over 287 Cα[32]. In the METTL16:RNA structure, the substrate adenine binds in a similar plane and position as the substrate adenine in BA4 (b) (Figure 5A). In METTL16, the adenine is swapped 180° relative to the BA4(b) base, but the N6-atom to be methylated are located only 1.5 Å apart. Compared to the 4.0 Å distance between the N6-atom and δ N-atom in BA4(b), the N6-atom in METTL16 is at a distance of 4.6 Å to the corresponding S-atom of a modelled SAH molecule (Figure 5A). Apart from similarities at the level of the substrate base, the RNA from METTL16:RNA does not model well onto RlmJ with multiple clashes observed with the core Rossmann-fold. Taken together, a comparison of BA4(b) with RNA-bound METTL16 indicates, that the substrate part of BA4(b) could bind METTL16 in the position of the RNA substrate, and might thus be used broadly to study RNA binding in  $m^6A$  MTases.





**Figure 4.** Binding conformations adopted by BA4. (A) RlmJ protein bound to the folded conformation of BA4 (BA4(a)) (red). RlmJ:SAH is aligned with RlmJ:BA4 and shown in yellow. The parts of BA4(b) involved in  $\pi$ -stacking are indicated, as well as the parts of the compound corresponding to the natural substrate -and cofactor. The 1.7 Å displacement of the central  $\delta$  N-atom from the expected position is indicated as a dashed black line. (B) As for A, but presenting the extended conformation of BA4 (BA4(b)) (blue). The point of rearrangement from S-configuration in SAH to R-configuration in BA4(b) is indicated, along with the resulting degree of rotation ( $120^\circ$ ) for the cofactor part of BA4(b). The parts of the compound corresponding to the natural substrate -and cofactor are indicated. The distance between the N6-atom of adenine and the  $\epsilon$  C-atom from the cofactor part (dashed black line) is given in Å. (C) Fo-Fc of BA4(a) (top, red) and BA4(b) (bottom, blue) contoured at  $2.0 \sigma$ . The substrate - and cofactor parts are indicated for clarity. (D) RlmJ bound to BA4(b) (blue). Hydrogen bonds are indicated as dashed lines and distances are shown in Å between both the N1-/N6-atoms of the BA4(b) substrate base, the cofactor part of BA4(b) that is bound similarly to SAH, and the catalytically important residues; K18 and D164 (stick representation, purple). BA4(b) is shown in blue stick representation. (E) Superposition of RlmJ:BA4(b) (blue) with RlmJ:SAH (yellow). The substrate base from BA4(b) is fitted into the closed conformation of RlmJ, which is adopted when the protein binds to SAH. Dashed lines indicate the hydrogen bonds formed between different RlmJ residues (stick representation, yellow) in the closed conformation around the substrate base from BA4(b). Distances are shown in Å.

In addition to resembling each other, the active site of  $m^6A$  RNA MTases also align well with active site components of  $m^6A$  DNA MTases. Thus, the bisubstrate analogues might also be used to study substrate binding in this subfamily of proteins. A DALI search comparing RlmJ pairwise with the  $m^6A$  DNA MTases T4Dam (PDB: 1YFL) or M.TaqI (PDB: 1G38) yielded RMSD-values of  $3.2 \text{ \AA}^2$  and  $2.8 \text{ \AA}^2$  over 281 C $\alpha$ , respectively [32]. T4Dam and M.TaqI were previously crystallised in complex with substrate DNA, and one of the cofactor analogues; sinefungin or 5'-[2-(amino)ethylthio]-5'-deoxyadenosine (AETA), respectively. A superposition of their cofactor analogues (sinefungin, AETA) with SAH and BA4(b) in RlmJ reveals that the substrate adenine of BA4(b) locates in the same area as the substrate base in M.TaqI (Figure 5B) and T4Dam (Figure 5C), resembling most that of T4Dam. Interestingly, in these proteins the adenine base is bound in the same plane as in BA4(b), emphasizing the potential of BA4 to also mimic the substrate:cofactor bound state in DNA MTases, further broadening the applicability of this molecule in DNA binding studies.

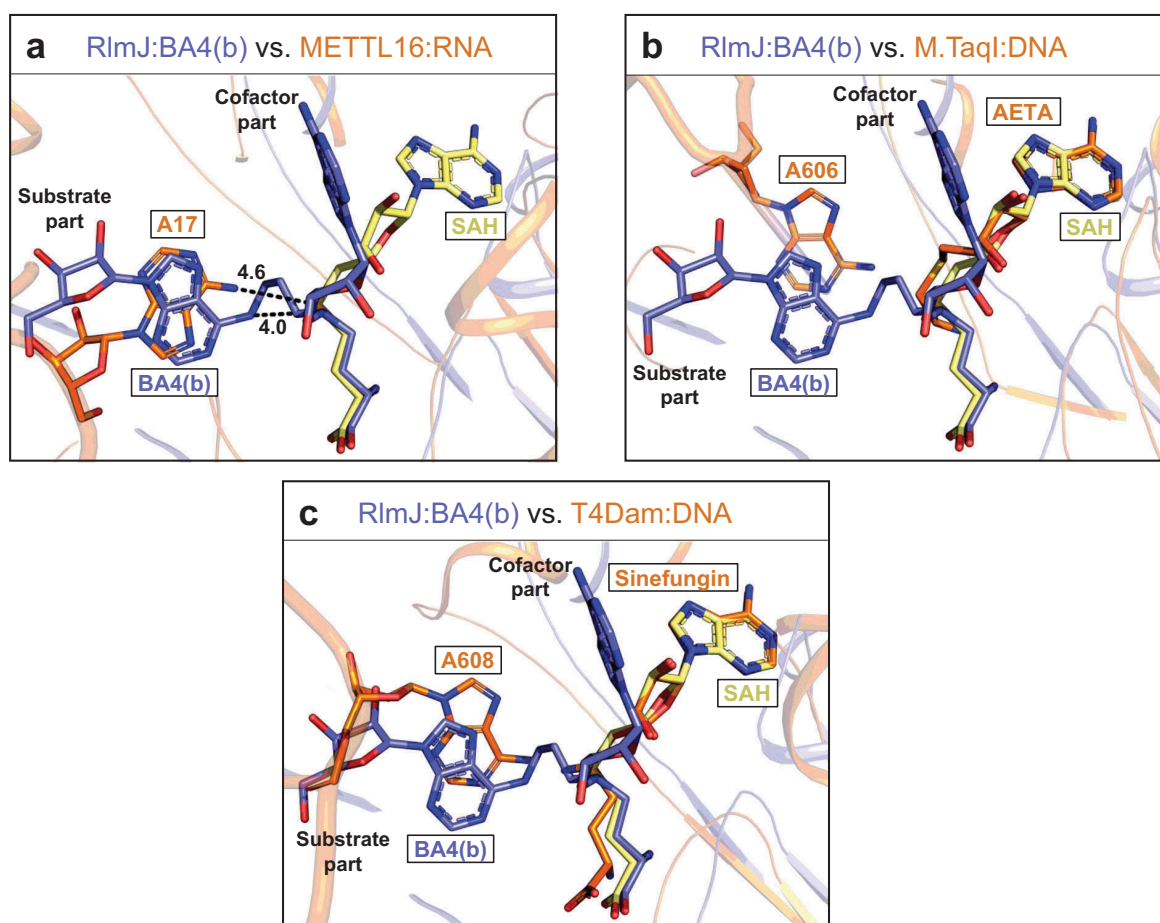
## Discussion

In this study, we successfully identify two bisubstrate analogues that bind to the active site of RlmJ: BA2 or BA4. ITC

data for RlmJ with these compounds showed  $K_D$ -values in the low micromolar range. The values for BA2 and BA4 were about 15-fold higher than that of sinefungin, but comparable to  $K_D$ -values found for the dual inhibitor DS-437 of 25 and 30  $\mu\text{M}$  against the human protein MTases hPRMT5:hMPE50 and hPRMT7, respectively [22]. Based on their corresponding  $IC_{50}$ -values of  $5.9 \pm 1.4 \mu\text{M}$  and  $6.0 \pm 0.5 \mu\text{M}$ , and the inactivity of DS-437 towards 29 other human protein-, DNA- and RNA MTases, this probe was deemed a promising template for the development of potent and selective inhibitors for hPRMT5 and hPRMT7. We thus hypothesize that the BA2 and BA4 compounds could similarly serve as template scaffolds for inhibitor design against  $m^6A$  RNA MTases.

Binding investigation with BA2, BA4 and the  $m^1A$  RNA MTase protein TrmK revealed a similar pattern for this protein, compared to RlmJ. Thus, inhibitory optimization of these compounds for  $m^6A$  RNA MTases should aim to increase not just affinity, but also specificity.

For optimization of affinity, some hints were provided from the Tm of RlmJ in the presence of each substrate analogue. Analogues which included the methionine part of the cofactor displayed a larger increase in protein thermostability, compared to analogues lacking this cofactor part. This indicates that the methionine part of the analogues interacts directly with the



**Figure 5.** Comparison of RlmJ:BA4(b) with RNA-bound  $m^6A$  RNA and DNA MTases. (A) Comparison of the position of the substrate base of BA4(b) (blue) from RlmJ:BA4 and the corresponding base (A17) in the human homologue METTL16 co-crystallised with an RNA substrate (PDB: 6DU4) (orange). SAH (yellow) from RlmJ:SAH, aligned with RlmJ:BA4, is shown for comparison. The methylated bases and all cofactors are shown in stick representation. (B) As for A, but compared to the corresponding base (A606) from the DNA co-crystallised with  $m^6A$  DNA MTase M.TaqI (PDB: 1G38) bound to the cofactor analogue 5'-[2-(amino)ethylthio]-5'-deoxyadenosine (AETA) (orange). (C) As for A, but compared to the corresponding base (A608) from the DNA co-crystallised with  $m^6A$  DNA MTase T4Dam (PDB: 1YFL) bound to the cofactor analogue sinefungin (orange).

protein, as confirmed by our RlmJ:BA crystals structures. A similar larger increase was also consistently observed for the longer linker of three C-atoms (BA3,-4) compared to the linker of two C-atoms (BA1,-2), or a urea unit (BA5,-6). Comparison of the structure of BA2 containing a two C-atom linker (folded conformation) with those of BA4 containing a three C-atom linker (folded and extended conformation), indicates that a longer linker is required to allow the molecule to adopt the extended form. We thus suggest that future designs should include the SAM methionine group and a linker of at least three C-atoms length. A successfully optimized compound would ultimately be used either as a small-molecule drug, or as a cell-active chemical compound, to investigate the therapeutic potential of targeting N6-monomethylation pathways. Such a compound becomes even more interesting with the discovery of the human  $m^6A$  RNA MTase METTL16 and METTL3:METTL14 complex, all related to RlmJ in structure, as these targets have been associated with a number of cancers [18].

Structures of proteins in complex with RNA and cofactors are difficult to obtain for a number of reasons: (1) the often-low protein affinity for the modified base itself excludes the use of just single nucleotides such as AMP or GMP for

structural studies, (2) RNA-binding often involves concomitant binding of cofactors, complicating complex reconstitution, (3) the RNA has to be correctly folded and homogeneous for facile crystallisation, and (4) incubation in crystallisation trials requires completely RNase free conditions. SAM-dependent MTases often co-crystallise easily with the cofactor. Therefore, directly linking the substrate base to this moiety at the point of nucleophilic attack to mimic the substrate:cofactor bound state, facilitates structural studies by increasing RNA substrate base affinity, reducing flexibility, and by-passing RNase contamination issues. Using such linked molecules, we solved the co-crystal structure of RlmJ with either of two compounds: BA2 or BA4. Structure analysis revealed that the adenine base in an extended conformation of BA4 likely binds in the real substrate binding pocket for substrate RNA. Thus, this molecule might be used more generally as a tool to determine the substrate binding site in other  $m^6A$  RNA MTases. Structural similarities between  $m^6A$  RNA and DNA MTases suggests that the here tested bisubstrate analogues could also be used for  $m^6A$  DNA MTases, further broadening the applicability of these compounds.

The newly synthesized bisubstrate analogues [24] appear with both the R- and S-configuration with respect to the  $\delta$  N-atom. The cofactor adenosine of the extended form of BA4 binds outside of the natural cofactor binding pocket due to an R/S-configurational rearrangement. Using a BA containing a carbon instead of the  $\delta$  N-atom would allow to synthesize BA with S-configuration and should favor the good positioning of the cofactor moiety into the catalytic site of the methyltransferase. A C-atom is already present in the only known RNA MTase inhibitor; sinefungin, which displayed the highest  $T_m$  for RlmJ, of all tested compounds. The characteristics of the atom at this position (the  $\delta$ -atom) was shown throughout our experiments to be generally important for protein thermostability, and thus likely also for the  $K_D$ .

Overall, we present here the first utilization of bisubstrate analogues to study substrate recognition of  $m^6A$  MTases. The substrate parts of these molecules occupy the substrate binding pockets and might be used to study substrate binding and mechanisms in protein throughout the  $m^6A$  RNA and DNA MTase families. Chemistry that allows coupling of the cofactor to different positions in the RNA base or sugar, would facilitate the production of a full co-crystallisation library of MTase bisubstrate analogues, matching each family of proteins catalyzing post-transcriptional modifications. Such a library would strongly forward the fields understanding of substrate recognition and enzymatic mechanisms of these protein families.

## Methods/materials

### Cloning, expression and purification of RlmJ and TrmK

DNA fragments encoding the entire proteins of RlmJ (residues 1–280) or TrmK (residues 1–245) were subcloned into the pET15b vector bearing an N-terminal His<sub>6</sub>-tag followed by a thrombin protease cleavage site. Plasmids were transformed into *E. coli* BL21(DE3) competent cells, cultured in Lysogeny Broth at 37°C, and protein expression was induced with 0.5 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside for 3 h growth at 37°C. Cell pellets from 1–2 L culture were lysed by sonication, clarified by centrifugation, and the clarified lysate was applied to immobilized metal affinity column (IMAC) for purification. After IMAC, TrmK purity necessary for crystallisation assays was reached (Supplementary Figure S4) and TrmK was concentrated before storage at –80°C in a buffer of 50 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol, 1 mM dithiothreitol and 1 mM ethylenediaminetetraacetic acid. Further purification of RlmJ was performed by size exclusion chromatography (SEC) on Superdex S75 PG (GE Healthcare). Parts of the expressed protein was further treated with Thrombin protease for 4 h at 22°C to remove the His<sub>6</sub>-tag and purified by SEC on Superdex S75 PG (GE Healthcare). After these steps, RlmJ purity necessary for crystallisation assays was reached (Supplementary Figure S4). RlmJ was concentrated and stored at –80°C in a buffer of 20 mM Tris pH 7.0, 150 mM NaCl, 5% glycerol and 5 mM  $\beta$ -mercaptoethanol.

### Analytical size exclusion chromatography (analytical SEC)

100  $\mu$ L protein sample of 90  $\mu$ M for RlmJ and 30  $\mu$ M for TrmK was injected onto a 24 mL Superdex Increase S75 10/300 GL column (GE Healthcare). MW standards from Bio-Rad containing thyroglobulin (670 kDa),  $\gamma$ -globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (13.5 kDa) were used for calibration. MWs for RlmJ and TrmK were determined from a linear plot of LOG [10](MW) with the MW of each Bio-Rad standard proteins against their elution volume.

### Differential scanning fluorimetry (DSF)

DSF was performed in a 96-well plate using a CFX96Touch real-time PCR detection system (Bio-Rad) with excitation and emission filters of 450–490 and 515–530 nm, respectively. Each well consisted of 2  $\mu$ L protein in a buffer of 20 mM HEPES pH 7.5, 150 mM NaCl to a final concentration of 5  $\mu$ M, 2  $\mu$ L of SYPRO ORANGE diluted 5000-fold in buffer from the manufacturer's stock (Invitrogen), and (if applicable) 2  $\mu$ L ligand to 1 mM. Fluorescence intensities were measured from 25 to 85°C with a ramp rate of 1°C/min.  $T_m$  was determined by curve-fitting using GraphPad Prism v.5.01 software [25]. Data were evaluated using a standard P-test according to standard procedure performed using the GraphPad Prism v.5.01 software.

### Isothermal titration calorimetry (ITC)

ITC measurements for the binding affinities between RlmJ and SAH, sinefungin, BA2 or BA4 were performed at 25°C using an ITC200 titration calorimeter (MicroCal/Malvern Panalytical). The molecules were dissolved in protein buffer of 20 mM Tris pH 7.0, 150 mM NaCl, 5% glycerol and 40  $\mu$ L of this was placed in a syringe. The molecules were titrated into 200  $\mu$ L of the protein in the cell. The first injection (0.2  $\mu$ L) was followed by 23 injections for SAH and sinefungin and 19 injections for BA2 and BA4, each of 1.6  $\mu$ L or 2  $\mu$ L, respectively. The heat of dilution for the molecules was measured for background subtraction. The titration curves were analyzed using the Origin software (MicroCal) with a single binding-site model, after exclusion of the first injection point. Standard errors were estimated from the data spread and from the uncertainty of the titrant concentration determination as previously described [33]. Dissociation constant ( $K_D$ ) -and stoichiometry (N) values are reported as mean  $\pm$  standard deviation (SD).

### Crystallisation, data collection, and structure determination

Crystals of RlmJ (with BA2, BA4) or His<sub>6</sub>-RlmJ (with SAH) were grown by vapor diffusion at 20°C, from sitting drops composed of 200 nL of protein and 40  $\mu$ L of reservoir solution. For His<sub>6</sub>-RlmJ bound to SAH, the protein concentration was 13 mg/ml; pre-incubated with 760  $\mu$ M SAH and crystals grew with a reservoir solution containing 0.1M KSCN, 30%



(w/v) polyethylene glycol methyl ether (PEG MME) 2000. RlmJ bound to BA2 crystallised with a protein concentration of 9 mg/ml, pre-incubated with 2.2 mM BA2 in a reservoir solution containing 0.1M BIS-Tris pH 6.5, 25% (w/v) PEG3350. RlmJ bound to BA4 crystallised with a protein concentration of 9 mg/ml, pre-incubated with 2.7 mM BA4 in a reservoir solution of 0.1 M MMT pH 6.0, 25% (w/v) PEG1500. For TrmK bound to SAH, crystals were grown by vapor diffusion at 20°C, from sitting drops composed of 150 nL of protein and 40 µL of reservoir solution, with a protein concentration of 1.1 mg/ml, pre-incubated with 2 mM SAH in a reservoir solution of 0.2 M Li<sub>2</sub>SO<sub>4</sub>, 0.1 M HEPES pH 7.5, 25% (w/v) PEG3350. Crystals were cryoprotected with reservoir solution supplemented with 15% (v/v) glycol, and flash-cooled in liquid nitrogen. Diffraction data were collected on the Synchrotron Soleil beamline PX2 to 1.39 Å for RlmJ:BA2 and 2.10 Å for RlmJ:BA4, or at the European beamline ID23-2 to 1.61 Å for RlmJ:SAH and 2.36 Å for TrmK:SAH. Phases were determined by molecular replacement in PHASER [34] in the CCP4 Suite of programs [35], using an existing RlmJ structure as search model (PDB: 4BLV) for RlmJ complexes, and TrmK from *Bacillus subtilis* (PDB: 6Q56) for TrmK. Modelling and refinement were carried out using Refmac [36], COOT [37] and Phenix [38]. Density was visible for all residues aside from the tag and loopy parts covering residues 52–58 and 231–233 in chain A of RlmJ:SAH, residues 52–58 in chain B of RlmJ:SAH, residues 52–56 in RlmJ:BA2, and residues 1–2 and 53–57 in all chains of RlmJ:BA4.

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## Disclosure statement

No potential conflict of interest was reported by the authors.

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## Data Availability

Atomic coordinates and structure factors for the reported crystal structures have been deposited in the Protein Data Bank (PDB) under accession number 6QE5, 6QE0, 6QDX, 6QE6.

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