Co-crystal structures of the protein kinase haspin with bisubstrate inhibitors

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Haspin is a mitotic protein kinase that is responsible for the phosphorylation of Thr3 of histone H3, thereby creating a recognition motif for docking of the chromosomal passenger complex that is crucial for the progression of cell division. Here, two high-resolution models of haspin with previously reported inhibitors consisting of an ATP analogue and a histone H3(1–7) peptide analogue are presented. The structures of the complexes confirm the bisubstrate character of the inhibitors by revealing the signature binding modes of the moieties targeting the ATP-binding site and the protein substrate-binding site of the kinase. This is the first structural model of a bisubstrate inhibitor targeting haspin. The presented structural data represent a model for the future development of more specific haspin inhibitors.

1. Introduction

Protein kinases (PKs) catalyse phosphorylation, a ubiquitous reversible protein modification serving as a switch that determines the structure and properties and hence the activity profile of the phosphorylatable protein. The family of >538 human PKs can be divided into ten groups based on the similarity of their primary structures and modes of regulation (Cheng *et al.*, 2014; Manning *et al.*, 2002), whereas the ‘outliers’ (termed atypical or other PKs) possess structural features that are remarkably different from the canonical PKs (Zeqiraj & van Aalten, 2010).

Haspin (haploid germ cell-specific nuclear protein kinase) was initially considered to be an inactive pseudokinase based on homology alignment of its primary sequence with the well known catalytically important motifs of canonical PKs. Haspin lacks the DFG triad of the ATP/Mg²⁺-binding motif (replaced with DYT), the conserved Lys residue situated at the catalytic loop (replaced with His) and the APE motif of the activation segment (Higgins, 2001). The first co-crystal structures of the catalytic domain of haspin as an apoenzyme or in complex with ATP site-targeting compounds (AMP, 5-iodotubercidin *etc.*) shed light on the characteristic features of the spatial structure of this PK (Eswaran *et al.*, 2009; Villa *et al.*, 2009). The insertions in the usual bilobal core of PK involved the uIH helix and connecting loops (constituting an additional N-lobe layer), hairpin $\beta 7'–\beta 7''$ (packing against the C-lobe), strand $\beta 9'$ (forming an antiparallel sheet with $\beta 9$ at the tip of the activation segment) and the large helix αAS (replacing the usual APE motif and the *P*+1 loop). Additionally, the αG helix, which usually constitutes part of the substrate-binding

site, was excluded. Despite its atypical structure, haspin is highly active, and at least one of its natural substrates has been identified, represented by histone H3 (Dai *et al.*, 2005). Haspin is responsible for phosphorylation of the Thr3 residue of histone, thus creating a docking site for survivin, which as part of the chromosomal passenger complex (also involving borealin, INCENP and the protein kinase Aurora B) can then coordinate mitotic processes (Kelly *et al.*, 2010; Wang *et al.*, 2010, 2012). The recently resolved co-crystal structure of haspin with histone H3(1–7) provided further three-dimensional insight into the functioning of this PK by showing

the unique bent binding mode of the substrate, in which the backbone of the histone performed a 180° turn (Maiolica *et al.*, 2014).

Since the discovery of the catalytic activity of haspin, several small-molecular-weight scaffolds have emerged that serve as inhibitors of this PK. The latter compounds comprise ATP site-targeting compounds (De Antoni *et al.*, 2012; Huertas *et al.*, 2012; Patnaik *et al.*, 2008), including 5-iodotubercidin as mentioned above, as well as bisubstrate scaffolds consisting of both ATP site-targeting and substrate site-targeting fragments (Kestav *et al.*, 2015). In previous work, we

Figure 1

ARCs target both catalytically important binding sites of haspin. (a) Chemical structures of ARCs with functional fragments indicated. (b, c) Electron-density maps for the bound ligands. Shown are $|F_o| - |F_c|$ OMIT maps contoured at 3.0σ for ARC-3353 (b) and ARC-3372 (c). (d) Co-crystal structure of ARC-3353 with haspin; the kinase catalytic domain is depicted as a green cartoon and the inhibitor is shown in ball-and-stick representation (C atoms orange, O atoms red, N atoms blue). (e) Co-crystal structure of ARC-3372 with haspin; the catalytic domain is depicted as a cyan cartoon and the inhibitor is shown in ball-and-stick representation (C atoms magenta, O atoms red, N atoms blue). In (d) and (e) a red circle indicates the position of the ATP analogue moiety of the inhibitor in the ATP-binding site of haspin and an orange trapezoid shows the positioning of the histone-derived peptide moiety of the inhibitor located in the protein substrate-binding site of haspin.

described the development and biochemical characterization of such compounds as well as their effects on histone phosphorylation in HeLa cells (Kestav *et al.*, 2015). Here, we report co-crystal structures of the previously published bisubstrate scaffolds with haspin, and compare these with the previously published structures of haspin with AMP (PDB entry 3dlz; Eswaran *et al.*, 2009) and of haspin with 5-iodotubercidin and histone H3(1–7) (PDB entry 4ouc; Maiolica *et al.*, 2014).

2. Materials and methods

2.1. Protein production

Human recombinant haspin protein (residues 470–798) with a TEV-cleavable N-terminal His₆ tag was produced and purified according to previously published protocols (Chaikuad *et al.*, 2014; Eswaran *et al.*, 2009). The experimental *m/z* of the protein was determined by LC-MS using an Agilent LC/MSD TOF spectrometer, confirming the mass of the unphosphorylated protein.

2.2. Crystallization

Purified haspin with an N-terminal His₆ tag was concentrated to 12 mg ml^{−1} and mixed with the inhibitors at a final concentration of 1 mM. Complex crystals were grown using the sitting-drop vapour-diffusion method at 4°C with a reservoir solution consisting of 52–60% MPD, 0.1 M SPG pH 6.5–7.0.

2.3. Data collection and refinement

Crystals were flash-cooled in liquid nitrogen prior to data collection on beamline I04-1 at Diamond Light Source using an X-ray wavelength of 0.91741 Å. Diffraction data were processed with *MOSFLM* (Powell *et al.*, 2013) and subsequently scaled with *SCALA* (Evans, 2006) from the *CCP4* suite (Winn *et al.*, 2011). Initial structure solutions were obtained using *Phaser* (McCoy *et al.*, 2007) and the coordinates of the previously published haspin structure (Chaikuad *et al.*, 2014). The structures were subjected to model rebuilding in *Coot* (Emsley *et al.*, 2010) alternated with refinement using *REFMAC5* (Murshudov *et al.*, 2011). The geometric correctness of the final models was verified using *MolProbity* (Chen *et al.*, 2010).

3. Results and discussion

The conjugates ARC-3353 (*K_d* value of 170 nM towards haspin)¹ and ARC-3372 (*K_d* value of 150 nM towards haspin) chosen for co-crystallization with haspin consisted of an adenosine analogue (Adc) targeting the ATP-binding site of the PK and a histone H3(1–7)-like peptide targeting the protein substrate-binding site of the PK (Kestav *et al.*, 2015). These moieties were joined *via* a flexible linker (Ahx) and a chiral spacer (dAsp) that were expected to facilitate the

Table 1

Data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

	Ligand	
	ARC-3353	ARC-3372
PDB code	5htb	5htc
Data collection		
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Unit-cell parameters (Å, °)	<i>a</i> = 77.79, <i>b</i> = 78.89, <i>c</i> = 81.75, $\alpha = \beta = \gamma = 90.0$	<i>a</i> = 77.97, <i>b</i> = 78.90, <i>c</i> = 81.06, $\alpha = \beta = \gamma = 90.0$
Resolution (Å)	24.91–1.70 (1.79–1.70)	25.02–1.50 (1.58–1.50)
Unique observations	55487 (7999)	79693 (11332)
Completeness (%)	99.2 (99.0)	99.0 (97.6)
Multiplicity	4.1 (3.7)	5.6 (5.6)
<i>R</i> _{merge}	0.087 (0.552)	0.054 (0.262)
Mean <i>I</i> / σ (<i>I</i>)	8.9 (2.2)	17.0 (5.9)
Wilson <i>B</i> value (Å ²)	17.7	14.0
Refinement		
<i>R</i> _{work} / <i>R</i> _{free} (%)	16.2/18.1	15.0/17.1
No. of atoms		
Protein	2710	2738
Ligand	91	182
Others	391	426
<i>B</i> factors (Å ²)		
Protein	23	20
Ligand	28	27
Others	36	34
R.m.s.d., bonds (Å)	0.016	0.016
R.m.s.d., angles (°)	1.6	1.6
Ramachandran statistics		
Favoured (%)	98.83	98.56
Allowed (%)	1.17	1.44
Disallowed (%)	0	0

correct positioning of the inhibitor fragments in the corresponding binding sites of haspin. The only structural difference between the two compounds was represented by a functional group located at the C-terminus of the chiral spacer: in ARC-3353 the C-terminus was amidated, whereas in ARC-3372 it was in the form of a carboxylic acid (Fig. 1*a*).

Overall, the co-crystal structures of haspin with both ARC inhibitors were determined to high resolution, and the presence of both inhibitors was confirmed by unambiguous electron-density maps (Table 1; Figs. 1*b* and 1*c*). The complex importantly confirmed the bisubstrate character of the inhibitors, with ARC-3353 displaying one binding confirmation in contrast to the two potential binding modes of ARC-3372 (Figs. 1*d* and 1*e*). In both structures the adenosine-analogue moieties of the inhibitors were positioned in the ATP-binding site of haspin as expected, with the characteristic hydrogen bonds formed between the N1 and N6 atoms of purine and the backbone of Glu606 and Gly608 from the PK hinge (Eswaran *et al.*, 2009; Figs. 2*a* and 2*c*). Similarly to the previously reported co-crystal of haspin with AMP, the hydroxyl groups of the ribose moieties of the inhibitors donated several hydrogen bonds to the side chain of Asp611 and the carbonyl of Gly653 (Eswaran *et al.*, 2009; Figs. 2*a* and 2*c*).

The presence of a flexible Ahx linker in the structure of the inhibitors, however, resulted in a major upward shift of the P-loop of haspin, accompanied by an overall movement of the N-lobe construction incorporating the uH helix and its connecting loops (residues 489–532), thus resulting in a more

¹ ARC-3353 was previously published as Compound 15 and ARC-3372 as Compound 16 (Kestav *et al.*, 2015).

open conformation of the PK (Figs. 3*a*, 3*b* and 3*c*). This differed from the previously observed conformations of haspin either in co-crystals with ATP-binding site-targeting compounds (PDB entry 3dlz) or in a co-crystal of the ternary complex incorporating 5-iodotubercidin and histone H3(1–7) (PDB entry 4ouc).

The C-terminal part of the linker moiety in ARC-3353 and ARC-3372 formed hydrogen bonds to the backbone of amino-acid residues at the tip of the P-loop of haspin (Val494 in the case of the ARC-3353 co-crystal and Phe495 in the case of the ARC-3372 co-crystal; Figs. 2*a* and 2*c*). This was analogous to the pattern reported previously for co-crystals of ARCs with

Figure 2

Interactions between ARCs and haspin. (a) Co-crystal structure of ARC-3353 with haspin; the kinase domain is depicted as a green cartoon and the inhibitor is shown in ball-and-stick representation (C atoms orange, O atoms red, N atoms blue). (b) Overlay of the ARC-3353 co-crystal structure with PDB entry 4ouc; backbone atoms of the peptidic part of ARC-3353 or histone H3(1–7) are shown. In ARC-3353 C atoms are orange and N atoms are blue; in histone H3(1–7) C atoms are grey and N atoms are blue. (c) Co-crystal structure of ARC-3372 with haspin; PK is depicted as a cyan cartoon and the inhibitor is shown in ball-and-stick representation (C atoms magenta, O atoms red, N atoms blue). (d) Overlay of the ARC-3372 co-crystal structure with PDB entry 4ouc; backbone atoms of the peptidic part of ARC-3372 or histone H3(1–7) are shown. In ARC-3372 C atoms are magenta and N atoms are blue; in histone H3(1–7) C atoms are grey and N atoms are blue. In (a) and (c) the amino-acid residues of haspin interacting with inhibitor are shown as sticks and are numbered; the hydrogen bonds are shown as dotted lines; a black circle shows the positioning of the C-terminus of the chiral spacer of the inhibitor and black arrows indicate the positioning of the side chain of the Lys residue corresponding to Lys(+1) in histone H3(1–7).

Figure 3

Conformational versatility of haspin. (a) Overlay of the co-crystal structures of ARC-3353 (green) and ARC-3372 (cyan) with PDB entry 3dlz (pink) and PDB entry 4ouc (grey). PK is shown as a cartoon. (b) Enlarged N-lobes from an overlay of the co-crystal structures of ARC-3353 (green) and ARC-3372 (cyan) with PDB entry 4ouc (grey). PK is shown as a cartoon and Phe495 from the P-loop of haspin is shown as sticks. (c) Enlarged N-lobes from an overlay of the co-crystal structures of ARC-3353 (green) and ARC-3372 (cyan) with PDB entry 3dlz (pink). PK is shown as a cartoon and Phe495 from the P-loop of haspin is shown as sticks. (d) Overlay of the co-crystal structure of ARC-3372 with PDB entry 3dlz. PK is shown as a ribbon and coloured according to temperature factor.

the catalytic subunit of cAMP-dependent PK (Lavogina *et al.*, 2009; Pflug *et al.*, 2010). Another striking feature of the co-crystals of ARCs with haspin was the positioning of the aromatic side chain of Phe495: in both cases it was displaced from its previously reported folded position under the P-loop (Figs. 3*b* and 3*c*). In the ARC-3353 co-crystal structure the phenyl ring of Phe495 stayed almost perpendicular to the P-loop, pointing down towards the C-lobe; in this way, a hydrophobic stacking interaction occurred between the amide bond following the linker of the inhibitor and the side chains of Phe495 and Lys527 of haspin. On the other hand, in the ARC-3372 co-crystal structure the phenyl ring of Phe495 was fully pushed out from its position under the P-loop typical of ATP-mimetic inhibitor complexes. Instead, it constituted part of a hydrophobic pocket lined by the side chains of Val494 and Lys527 of haspin and a side chain of a lysine residue of the peptidic portion of the inhibitor [corresponding to Lys(+1)² in histone H3]. The latter residue was present in two distinct conformations in the ARC-3372 co-crystal (as discussed in detail below), one of which contributed to the aforementioned hydrophobic pocket.

The peptide moiety of the inhibitor bound to the substrate-binding site of histone and showed the characteristic U-turn, with the lysine residue corresponding to Lys(+1) of histone

positioned at the 'tip' of the turn (Maiolica *et al.*, 2014; Figs. 2*b* and 2*d*). The binding conformation of rest of the peptide was, however, quite different from the previously reported crystal structure with PDB code 4ouc. In PDB entry 4ouc the N-terminal Ala(−2) of the substrate peptide was positioned in the small surface cavity of haspin, with a hydrogen bond formed between the N-terminal primary amine group and Glu613 of the PK. In the ARC complexes the corresponding Ala of the inhibitors was not bound to this haspin pocket, but rather pointed downwards and formed hydrogen bonds to the side chain of Asn718 (Figs. 2*a* and 2*c*). Still, the new surrounding of this Ala residue was sterically quite tight, confirming the previous observation that modifications in this region of the inhibitor are poorly tolerated by haspin.

This repositioning of Ala was probably caused by different positioning of the following Arg residue [corresponding to Arg(−1) in histone] in both the ARC-3353 and ARC-3372 co-crystals compared with PDB entry 4ouc. In the latter, Arg(−1) resides inside the deep hydrophilic pocket of the N-lobe of haspin, with its side chain protruding to form a hydrogen bond to Asn588 from the upper layer. In the co-crystals of ARCs with haspin, the corresponding Arg was pointing under the arch formed by the linker to form charge-assisted hydrogen bonds to Asn654 and Asp687 (Figs. 2*a* and 2*c*). This conformation was made possible by the more open conformation of the kinase in both new co-crystals and was further facilitated by an intramolecular charge-assisted hydrogen bond in the ARC-3372 co-crystal. The latter hydrogen bond between the

² Numbers in parentheses indicate the positioning of the given residues in histone relatively to the Thr residue that can be phosphorylated by haspin; a minus sign shows positioning to the N-terminus and a plus sign to the C-terminus from the phosphoryl acceptor.

C-terminal carboxylic group of the chiral spacer and the Arg residue of the peptidic part of the inhibitor also caused a movement of the C-terminus of the chiral spacer closer to Arg in the ARC-3372 co-crystal compared with the bound conformation of ARC-3353 (Figs. 2*a* and 2*c*).

Such a positioning of Arg in the ARCs dictated the binding patterns of the rest of the peptidic moieties. In ARC-3353, where the C-terminus of the chiral spacer was not moved towards Arg, the C-terminal amide instead formed an intramolecular hydrogen bond to the carbonyl of the Lys residue corresponding to Lys(+1) of histone. This in turn resulted in suitable positioning of the side chain of Lys for the development of charge-assisted hydrogen bonds to Asp707 and Asp709 of haspin: the 'signature positioning' also observed for histone H3(1–7) in PDB entry 4ouc (Fig. 2*a*). In the case of ARC-3372, however, such a direct stabilizing hydrogen bond between the C-terminus of the chiral spacer and the carbonyl of Lys was missing, which then enabled a different binding conformation of Lys. Namely, the latter protruded towards the N-lobe of the PK, with the alkyl part of its side chain participating in the aforementioned hydrophobic pocket with Val494, Phe495 and Lys527, and its side-chain amine group forming a hydrogen bond to Ser524 of haspin (Fig. 2*c*). Overall, the importance of the C-terminus of the chiral spacer for creating intramolecular and intermolecular hydrogen-bond patterns that has been unveiled in this work explains the fact that its exclusion has been tolerated by haspin in previous studies (Kestav *et al.*, 2015).

The different positioning of the Arg residue in ARC co-crystals with haspin also resulted in a different binding pattern of the C-terminal part of the histone-like peptide which was sterically prohibited in PDB entry 4ouc. In the latter, the side chain of the Gln(+2) residue made contacts with the amide bond between Gly713 and Asp714 from the activation loop of haspin, and the rest of the histone H3(1–7) sequence was tilted towards the small lobe of the PK. In the co-crystals with the ARCs, however, the corresponding Gln residue together with the rest of the peptidic part of inhibitor is folded upwards, facing the solvent (Fig. 2). In ARC-3353, owing to the fixed position of the Lys residue discussed above, the peptidic part of the inhibitor was located closer to the N-lobe of the PK, and a hydrogen bond was formed between the side chain of the Thr residue of the inhibitor [corresponding to Thr(+3) of histone] and the side chain of Asn588 of haspin (Fig. 2*a*). In ARC-3372, owing to one of the possible conformations of Lys in which it protruded towards Ser524 of haspin, the C-terminal part of the peptidic fragment of the inhibitor adopted a different conformation, resulting in the formation of a hydrogen bond between the side chain of Thr and the carbonyl of Asn522 of haspin (Fig. 2*c*).

The temperature factors of the new ARC co-crystals were generally in the same range as those reported for PDB entry 4ouc. Compared with PDB entry 4ouc, some destabilization of the N-terminus of haspin (residues 471–481) and a portion of the activation loop (residues 707–709) was evident. On the other hand, in the ARC co-crystals the regions corresponding to the N-terminal part of the α C helix of haspin (residues 528–

531), the loop between the β 9 and β 9' strands (residues 695–697) and the loop preceding the α X helix (residues 715–717) were slightly more fixed. Compared with PDB entry 3dlz, large portions of the N-lobe of haspin (residues 471–531 and 568–595) and its activation loop (694–716) were more fixed in the ARC co-crystals (Fig. 3*d*).

4. Conclusion

With the aid of two new co-crystal structures, we confirmed the bisubstrate character of the haspin-targeting inhibitors ARC-3353 and ARC-3372 developed in previous studies and demonstrated that minor changes in the structures of the inhibitors can lead to a significant rearrangement of the spatial structure of the inhibitor–enzyme complex. The co-crystals indicated unique features of the bound conformations of the inhibitors associated with the positioning of the linker chains (resulting in an overall opening of the catalytic cleft between the two lobes of the PK) and the development of intermolecular and intramolecular hydrogen-bond networks by the C-termini of chiral spacers that affected the interactions of the peptidic parts of inhibitors with haspin. In this way, the new crystal structures pinpointed the fact that the mechanistic approach to bisubstrate inhibitor design, in which the linker is only expected to serve as a flexible linking chain (that minimally intervenes in the binding of site-targeting fragments), is overly simplistic. We expect that the present work will provide useful guidance for 'precision-targeting' in the development of PK inhibitors, especially compounds that bind to protein–protein interfaces.

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