ELSEVIER

Haspin: a promising target for the design of inhibitors as potent anticancer drugs

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Protein kinases constitute a large group of enzymes in eukaryotes and have an important role in many cellular processes. Several of these proteins are active kinases, such as haploid germ cell-specific nuclear protein kinase (Haspin), an atypical eukaryotic protein kinase that lacks sequence similarity with other eukaryotic protein kinases. Haspin is a serine/threonine kinase that associates with chromosome and phosphorylates threonine 3 of histone 3 during mitosis. Haspin overexpression or deletion results in defective mitosis. It has been shown that Haspin inhibitors have potent anti-tumoral effects. Given that the only Haspin substrate is threonine 3 of histone 3, inhibition of Haspin might have fewer adverse effects compared with other anticancer agents. Here, we highlight the chemical structures and actions of currently known Haspin inhibitors.

Introduction

The protein kinases constitute a large group of enzymes in eukaryotes. These enzymes catalyse the transfer of the γ-phosphate of ATP (or GTP) to generate phosphate monoesters using the hydroxyl groups of many proteins. Depending on the proteins involved, this reaction results in either serine/threonine kinases or tyrosine kinases. These protein kinases are related by virtue of their homologous kinase domains and have important roles in many cellular processes (e.g., proliferation, gene expression, metabolism, motility, membrane transport, apoptosis, etc.); unsurprisingly, their misregulation often results in disease. Most eukaryotic protein kinases are members of the eukaryotic protein kinase superfamily. The kinase domain of protein kinases (250–300 amino acids) contains 12 conserved subdomains. The crystal structure of eukaryotic protein kinases shows that they have a bilobed structure (the smaller *N*-terminal lobe and the larger C-terminal lobe). The deep cleft between the two lobes is recognised as the site of catalysis. Three functions are attributed to the kinase domain: (i) binding and orientation of the ATP (or GTP) phosphate donor as a complex with the divalent cation; (ii) binding and orientation of the protein substrate; and (iii) transfer of the γ-phosphate from the ATP to the hydroxyl residue of the protein substrate [1,2]. In 1995, Hanks and Hunter proposed the first classification of eukaryotic protein kinases, based on their structural and functional properties [2]. In 2002, the classification was refined by Manning *et al*. based on the extracatalytic sequence similarity and biological function [3]. Several protein kinases lack sequence similarity with eukaryotic protein kinases and, thus, have been classified as inactive pseudokinases [3].

Haploid germ cell-specific nuclear protein kinase (Haspin) proteins are divergent members of the eukaryotic protein kinase family. Haspin (encoded by germ cell-specific gene 2: *Gsg2*) is found in many eukaryotic lineages (animal, fungi, and plants), and the protein and its mRNA were initially detected in male germ cells [4]. All proliferating cell lines express Haspin mRNA, and it has been detected in thymus, bone marrow, and foetal liver and, more weakly, in spleen, intestine, lung, and a variety of fetal tissues. Its expression is correlated with tissues that have signifi-

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GLOSSARY

Budding uninhibited by benzimidazole 1 (Bub1) and budding uninhibited by benzimidazole-related 1 kinases (BubR1) serine/threonine checkpoint kinases with important roles as kinetochore-microtubule attachment regulators.
Centromere protein A (CENP-A) a centromere-interacting protein (along with CENP-B, CENP-C1, CENP-D, CENP-E and CENP-F).
CDC2-like kinase 1 (CLK1) has an important role in the regulation of RNA splicing through phosphorylation of members of the serine and arginine-rich (SR) family of splicing factors.
Chromosomal passenger complex (CPC) comprises Aurora B, INCENP, Borealin, and Survivin; a key orchestrator protein of orderly mitotic exit and cytokinesis.
DYRK(1A, 1B, 2, 3) members of the dual specificity tyrosine-phosphorylation-regulated kinase family.
Highly expressed protein in cancer 1 (Hec1) located at the centromere during cell mitosis; has an important role in the spindle checkpoint pathway.
Protein kinase Ac (PKAc) catalytic subunit of cAMP-dependent protein kinase.
Repo-Man nuclear protein that is a specific regulatory subunit for PP1γ. Repo-Man disperses in the cytoplasm as cells enter prophase but relocalises to chromatin at anaphase onset. Repo-Man was identified as a complex responsible for inactivation of a regulator of chromosome architecture in anaphase.
Spindle assembly checkpoint (SAC) sometimes referred to as the 'mitotic checkpoint' or 'M-phase checkpoint'; a quality-control mechanism that prevents anaphase until all the chromosomes are stably attached to the spindle.

cant levels of cellular proliferation and differentiation [5]. Studies have confirmed Haspin as a serine/threonine kinase and a constitutively active enzyme.

Studies have shown that, during interphase, Haspin is autoinhibited by a conserved segment of basic residues (the Haspin basic inhibitory segment; HBIS) within the N-terminal domain, immediately upstream of the kinase domain. The kinase is reactivated in M phase by Cdk1 phosphorylation of the N terminus (Fig. 1). This phosphorylation leads to recruitment of Polo-like kinase-1 (Plk-1), which in turn further phosphorylates multiple sites at the N-terminal domain of Haspin. In addition, in human cells, the localisation of Aurora B kinase to the centromere creates a positive feedback loop that increases Haspin activity [6].

The crystal structure of Haspin reveals at least four peculiarities [5]: (i) the N-terminal lobe is entirely buried under an additional layer created by an N-terminal extension and two insertions; (ii) reorganisation of the activation segment contributes to the creation of an unusual substrate-binding site; (iii) an additional insertion between the β7 and β8 loop that contains two β-strands; and (iv) deletion of the αG helix.

During mitosis, Haspin localises predominantly to condensed chromosomes during mitosis, to centrosomes following nuclear envelope breakdown (NEBD), to spindle microtubules during metaphase, and to the midbody during telophase. The only know substrate of Haspin is histone H3. During mitosis, Haspin phosphorylates histone H3 at threonine 3 to form H3T3 ph [7]. Phosphorylated threonine 3 is detected on condensing chromosomes during prophase, prometaphase, and metaphase, is decreased during anaphase and is absent during telophase. Histone H3T3 ph creates a chromatin-binding site for survivin and recruits the **chromosomal passenger complex** (CPC; see Glossary) at inner centromeres during mitosis [8–12]. Haspin activity facilities the activation of Aurora B, a member of the CPC (Fig. 2). Aurora B kinase activity is necessary for full phosphorylation of Haspin during mitosis and stimulates H3T3 phosphorylation. It also acts to generate a positive feedback between Haspin and Aurora B and allows CPC accumulation on chromatin during mitosis [13]. The complex protein phosphatase 1γ(PP1γ)/**Repo-Man** induces indirect inhibition of Haspin by dephosphorylating of H3T3 at the end of mitosis [14,15].

Haspin overexpression or deletion results in defective mitosis. Inhibition of Haspin prevents normal chromosome alignment at metaphase, while Haspin overexpression results in a delay before metaphase. In addition, Haspin depletion leads to the loss of the cohesion association and activation of the spindle checkpoint, arresting mitosis in a prometaphase-like state [16].

Haspin inhibitors have antimitotic effects and might have fewer adverse effects than many other kinase inhibitors currently used as therapeutics because Haspin has only one known substrate (Thr 3 of histone 3) [17]. A previous study showed that Haspin inhibitors cause the displacement of Aurora B from inner centromeres, resulting in its diffuse distribution on chromatin [17] many other kinase inhibitors currently used as therapeutics.

Haspin inhibitors

Acridine derivatives

Using high-throughput screening (HTS), the compound LDN-192960, an acridine derivative, was identified as a potent kinase Haspin inhibitor with IC_{50} = 0.01 MM (Fig. 3) [18]. Based on the structure–activity relationships (SAR) of the acridine series, Cuny *et al*. [19] identified many Haspin inhibitors, the general structure of which is given in Fig. 3. All compounds showed good inhibitory activity again Haspin, while compound LDN-209929 was very selective for Haspin (180-fold selectivity versus **DYRK2**). LDN-209929 was reported to compromise the spindle checkpoint during mitosis when microtubules are severely disrupted [17]. However, the adverse effects highlighted with either acridine or tacrine, for example, do not support the use of this scaffold in the development of drugs for use in humans.

Beta carboline derivatives

Harmine and harmalol

Harmine is an indole alkaloid from the plant *Peganum harmala* with the pyrido[3,4-b]indole ring structure characteristic of β-carboline family alkaloids (Fig. 3), which includes harmaline, harman, and harmalol. These compounds exhibit psychoactive activity on the central nervous system (CNS), with hallucinogen adverse effects. Harmine has also been identified as an inhibitor of DYRK family kinases, with IC_{50} values of 0.03–0.35 MM reported for DYRK1A, and approximately 50-fold lower potency toward DYRK2 [20–23]. More recently, using time-resolved fluorescence resonance energy transfer (TR-FRET), Cuny *et al*. showed harmine

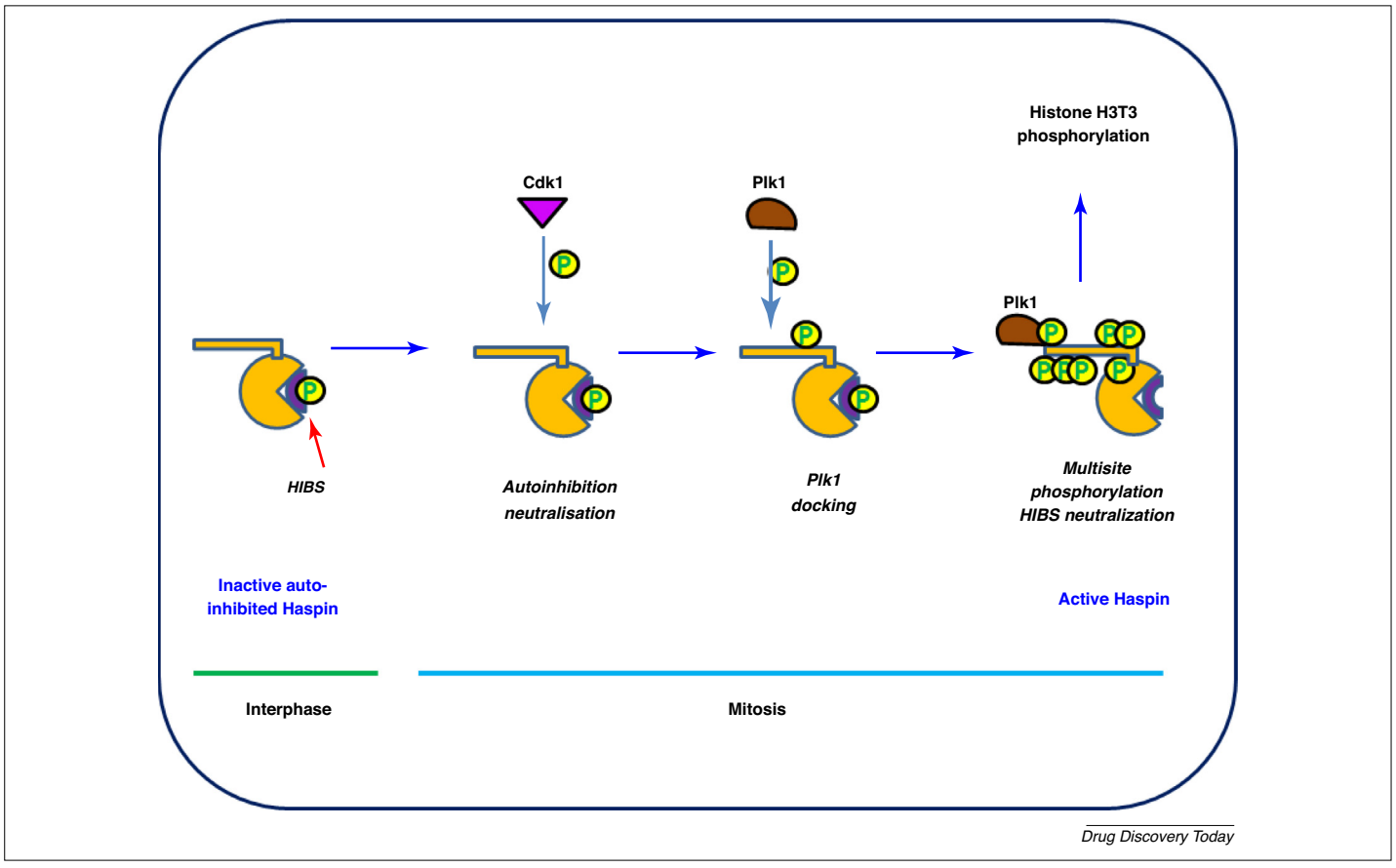


FIGURE 1

Haspin activation in mitosis. During interphase, the histone H3T3 kinase haploid germ cell-specific nuclear protein kinase (Haspin) is autoinhibited by a basic segment (Haspin basic inhibitory segment; HBIS). At the start of mitosis, Haspin undergoes priming phosphorylation of its N terminus by Cdk1, resulting in recruitment of polo-like kinase 1 (Plk1), which phosphorylates multiples sites at the Haspin N terminus, activating the protein.

and harmalol to be moderately potent Haspin inhibitors, with IC_{50} values of 0.59 and 0.77 MM, respectively [24]. The adverse effects of these compounds on the CNS limit interest in their use as therapeutics, although this structure could provide a useful scaffold for the design of optimised drug-candidates.

LDN-211898 and related compounds

In a SAR study of beta-carboline derivatives and by using the results of SAR studies carried out with the acridine series, compound LDN-211898 (Fig. 3) was identified as a lead compound. This compound has an IC_{50} value for Haspin of 0.10 μM and was very selective for Haspin (150-fold selectivity versus DYRK2) [24]. This study confirmed the beta-carboline scaffold as pharmacophoric structure that could be useful for the design of Haspin inhibitors. Interestingly, the lack of strict specificity towards Haspin (observed for LDN-211898 and some related compounds) could be advantageous, leading to compounds targeting simultaneously several kinases with an essential role during cell division.

Ribofuranosyl derivatives

5-ITu Initially, 5-iodo-7-β-D-ribofuranosyl-7*H*-pyrrolo[2,3-d]pyrimidin-4-amine (5-ITu; Fig. 4) was characterised as an adenosine kinase inhibitor by Wotring and Townsend [25]. Recently, it was demonstrated that 5-ITu, with an IC_{50} of 5–9 nM, has potent and selective inhibitory activity against Haspin *in vitro* [26–29]. 5-ITu targets the ATP-binding site of Haspin and causes dose-dependant displacement of the CPC subunits [17,29]. 5-ITu also compromised chromosome alignment and caused a dose-dependant decrease in mitotic (phospho)-protein monoclonal-2 phosphoepitopes. According to De Antoni *et al.*, 5-ITu potently inhibited Haspin but not Aurora B *in vitro*, and counteracted the centromeric localisation of the CPC without affecting the bulk of Aurora B activity in HeLa cells. Mislocalisation of Aurora B correlated with dephosphorylation of **Centromere protein A** (CENP-A), **Highly expressed protein in cancer 1 (Hec1)**, and **spindle assembly checkpoint** (SAC) override. In addition, 5-ITu impaired kinetochore recruitment of the mitotic checkpoint serine/threonine-protein kinases **Budding uninhibited by benzimidazole 1** (Bub1) and **budding uninhibited by benzimidazole-related 1 kinases** (BubR1), an effect that was reversed by concomitant inhibition of phosphatase activity. Forcing localisation of Aurora B to centromeres in 5-ITu also restored Bub1 and BubR1, but failed to rescue the SAC override. These results suggest that Haspin is required for a part of SAC signaling that is distinct from the centromere and kinetochore localisation of Aurora B and other proteins, whose localisation is Aurora B dependent; however, 5-ITu might inhibit a distinct SAC pathway in addition to that represented by the Haspin–Aurora B axis [29]. Thus, 5-ITu appears to be a useful tool for chemical biology investigations of Haspin activity in tumour development. However, the 'iodine' nature of the molecule could result in adverse

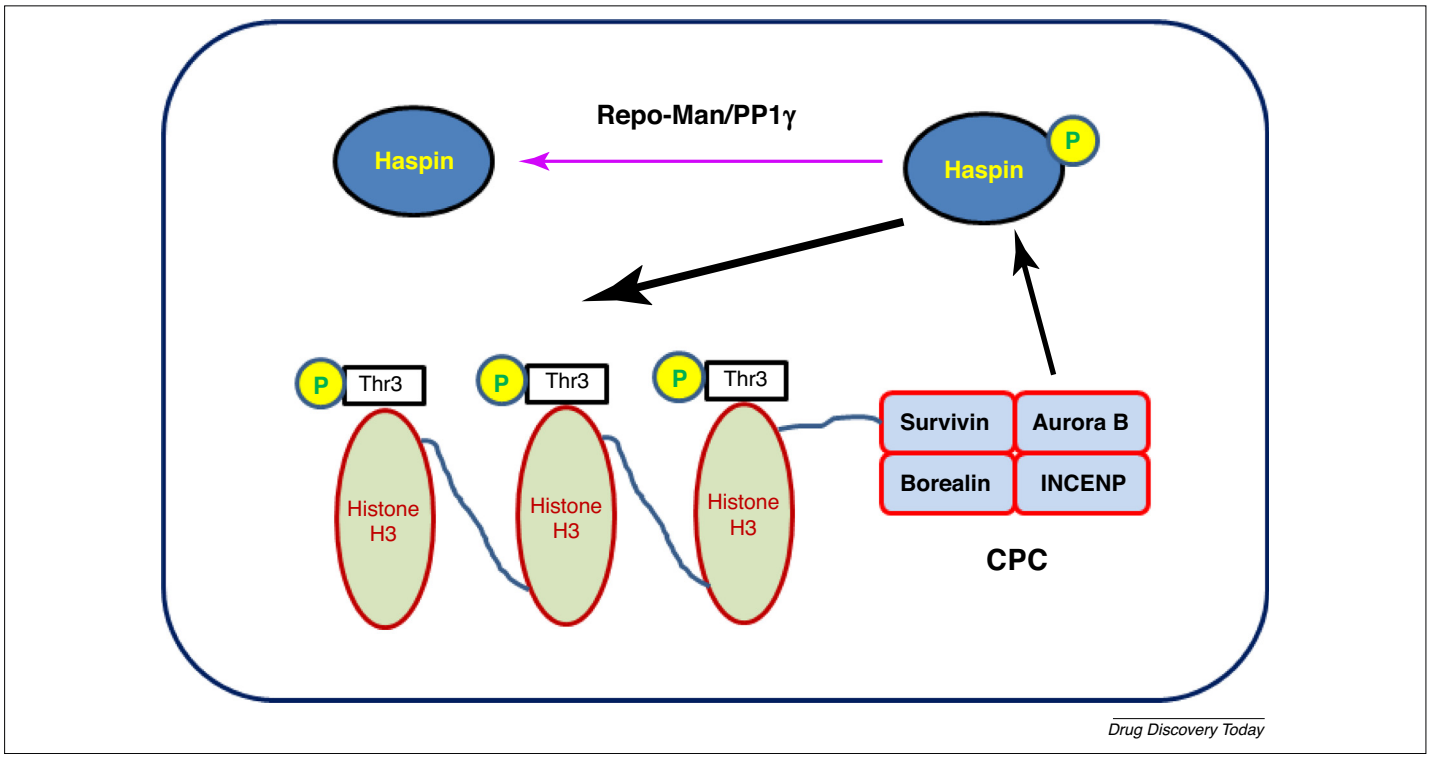


FIGURE 2

Relationships between Haspin and CPC. The nucleosome comprises four core histone proteins (H2A, H2B, H3, and H4) and is the primary building block of chromatin. Histone H3, one of the main histone proteins involved in chromatin in eukaryotic cells, has conserved N-terminal tail residues that undergo different post-translational modifications, such as phosphorylation. Four residues within the H3 N terminus are phosphorylated during mitosis: Thr3, Ser10, Thr11 and Ser28. Phosphorylation at Thr3, which is highly conserved among many species, is catalysed by Haspin and provides a chromatin-binding site for the recruitment of the chromosomal passenger complex (CPC). Aurora B, one of the four constituents of CPC, along with Survivin, Borealin and INCENP, phosphorylates Haspin, which creates a signal amplification loop. This contributes to CPC accumulation at centromeres and regulates mitosis and chromosome segregation. Haspin is then dephosphorylated by the protein phosphatase 1γ(PP1γ)/Repo-Man complex.

effects related to thyroid metabolism and, therefore, it is difficult to envisage such an iodo-scaffold being used in the design of molecules of clinical interest.

CHR-6494

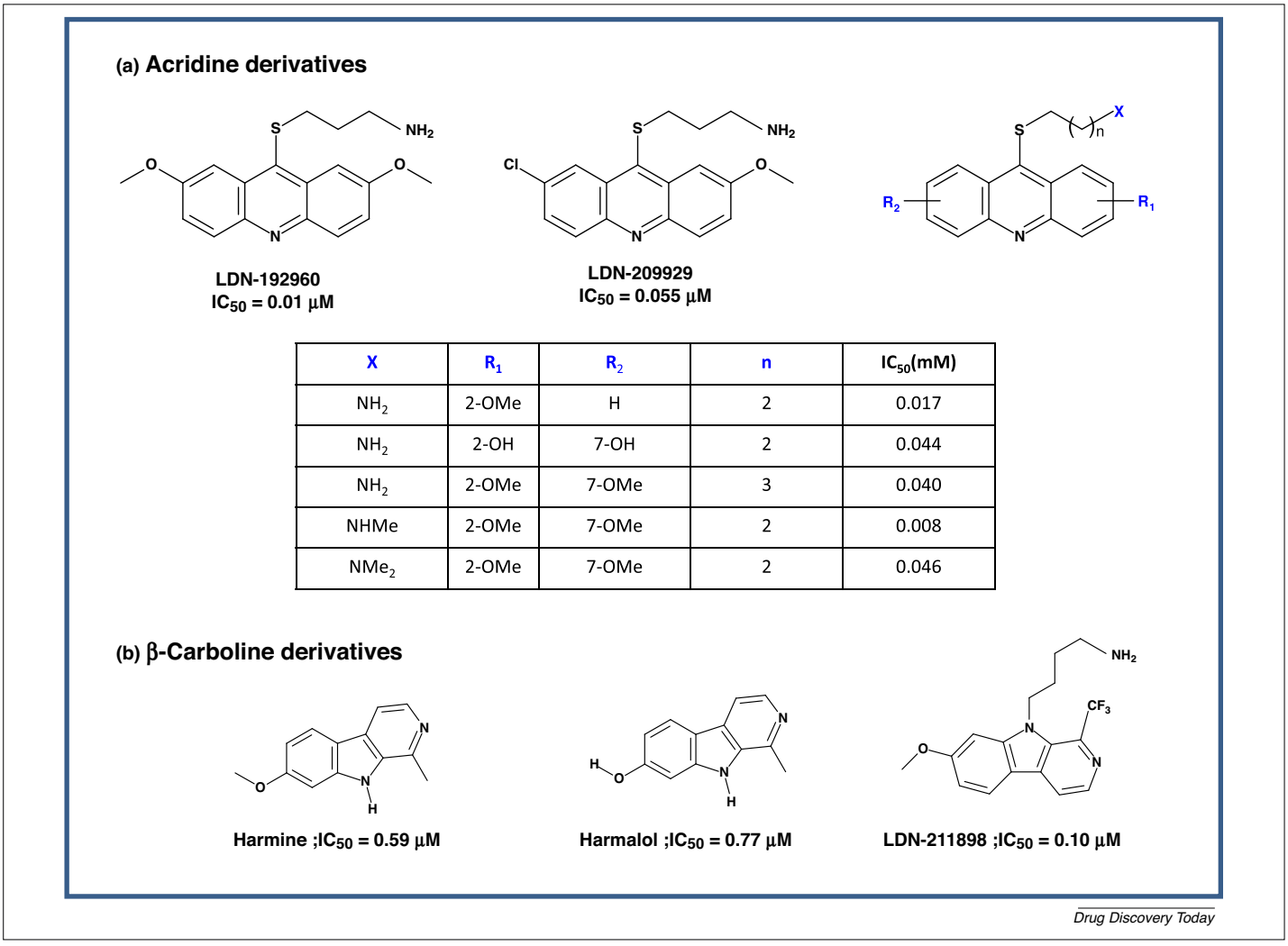
Compound 3-(1H-indazol-5-yl)-N-propylimidazo[1,2-b]pyridazin-6-amine (CHR-6494; Fig. 4) is a first-in-class Haspin inhibitor identified by HTS for kinase inhibitors, rather than being identified from a rational drug design strategy. This Haspin-selective inhibitor has an IC_{50} value of 2 nM and inhibited cancer cell growth dose dependently (473 nM for HeLa, 500 nM for HCT-116 and 752 nM for MDA-MB-231 cells). The compound also blocked H3T3 ph, resulting in an abnormal mitotic spindle and a large defect in chromosomal alignment and centrosomes. It also upregulated Bub1 and cyclin B1 and potently induced arrest in G2/M and subsequent apoptosis. In addition, CHR-6494 efficiently blocked basic fibroblast growth factor (bFGF)-induced vessel sprouting (by 70% at 1 μM in a chicken embryo aortic arch ring assay) and suppressed tumor growth in a HCT-116 xenografted mouse model (50 mg/kg, intraperitoneally) [30].

CHR-6494 appears to be a member of a new generation of antimitotic drugs targeting mitotic machinery other than microtubules, avoiding some of the adverse effects associated with, for example, taxanes, which occur because of the role of tubulin in many cellular functions in addition to mitosis. Thus, CHR-6494 constitutes an interesting tool for use in biological and antitumor investigations in addition to drug design, although further studies of its pharmacokinetic profile are required.

ARC-type bisubstrate inhibitors

Adenosine analogue-oligoarginine conjugate (ARC)-type bisubstrate inhibitors have been used in studies that involve the construction of chimeric structures (Fig. 4) comprising a fragment (nucleoside mimic) binding to the ATP-site of Haspin and a histone H3(1-7) peptide binding to the protein substrate site of a protein kinase, conferring the selectivity of these conjugates to this target [31]. The main expected advantage of bisubstrate inhibitors is their potential ability to generate more interactions with the target enzyme that could result in improved affinity and selectivity of the conjugates; several research groups have used this bisubstrate approach for the design and synthesis of inhibitors of various protein kinases [32].

As the nucleoside mimic section of these compounds, 5-ITu-like scaffolds were chosen, such as adenosine-4′-dehydroxymethyl-4′-carboxylic acid (Adc), 5-(2-aminopyrimidin-4-yl)-thiophene carboxylic acid (AMTH), and 6-aminohexanoic acid (Ahx). Based on this model, Uri and co-workers designed and developed first-generation ARC-type inhibitors of protein kinases, such as HER2, Aurora, CDK ROCK, or PIM-1, resulting in the identification of the reference compound ARC-902 (Fig. 4). More recently, studies on the Haspin/Histone H3 (1-7) co-crystal predicted two positions in the structure of the Histone H3(1-7) peptide to which



X	R_1	R_2	n	IC_{50}(mM)
NH_2	2-OMe	H	2	0.017
NH_2	2-OH	7-OH	2	0.044
NH_2	2-OMe	7-OMe	3	0.040
NHMe	2-OMe	7-OMe	2	0.008
NMe_2	2-OMe	7-OMe	2	0.046

FIGURE 3

Chemical structures of **(a)** acridine and **(b)** β-carboline compounds.

nucleoside mimics could be linked [33]. The first position is located near the N terminus of peptide and the second near to the phosphorylatable residue, Thr3.

These two aspects of this strategy resulted in the identification of two related series of new potent bisubstrate inhibitors of protein kinases, with a specific approach for targeting Haspin leading to the identification of ARC-3354 (Fig. 4) as lead compound. ARC-3354 has a subnanomolar Kd value toward Haspin and a 90-fold higher affinity toward Haspin than towards **protein kinase Ac** (PKAc), the basophilic reference kinase [31].

However, despite an excellent affinity and selectivity profile determined by *in vitro* evaluation, these compounds suffer an absence of data concerning their membrane-penetrative capability as well as their pharmacokinetics. Moreover, the peptidic character of these inhibitors could limit their development as drug candidates and, consequently, their therapeutic interest.

Concluding remarks

The cell cycle is an elaborate and evolutionarily conserved process in actively proliferating cells. Given that cell cycle aberrations are a hallmark of cancer, mitosis has been the target of anticancer strategies for decades; however, despite numerous mitosis-selective drug discovery strategies (e.g., microtubule-targeting agents, antimitotic kinases, and antimotor proteins; Table 1) and ensuing clinical trials, mitosis-targeted anticancer therapies have generally failed to translate their preclinical efficacy into clinical responses in human trials owing, in particular, to undesirable effects on nonproliferating cells and the emergence of drug resistance.

Here, we have discussed the development of inhibitors of Haspin, a serine/threonine kinase and a divergent member of the eukaryotic protein kinase family. During the past decade, many Haspin inhibitors have been identified and have validated this kinase as a target for anticancer drugs. Structures of Haspin inhibitors are very divergent but the established SARs and structure-selectivity relationships will be useful for the development of high-affinity Haspin inhibitors. These inhibitors could have potential therapeutic utility in cancer, with fewer adverse effects. In addition, such inhibitors could be combined with other mitosis-targeting drugs, to boost clinical efficacy. In addition, the key role of Haspin in many cellular processes and its important interactions with other mitotic kinases (e.g., Aurora B, Cdk1, Plk1, DYRKs, etc.) justifies the design of multitargeted mitosis inhibitors, in accor-

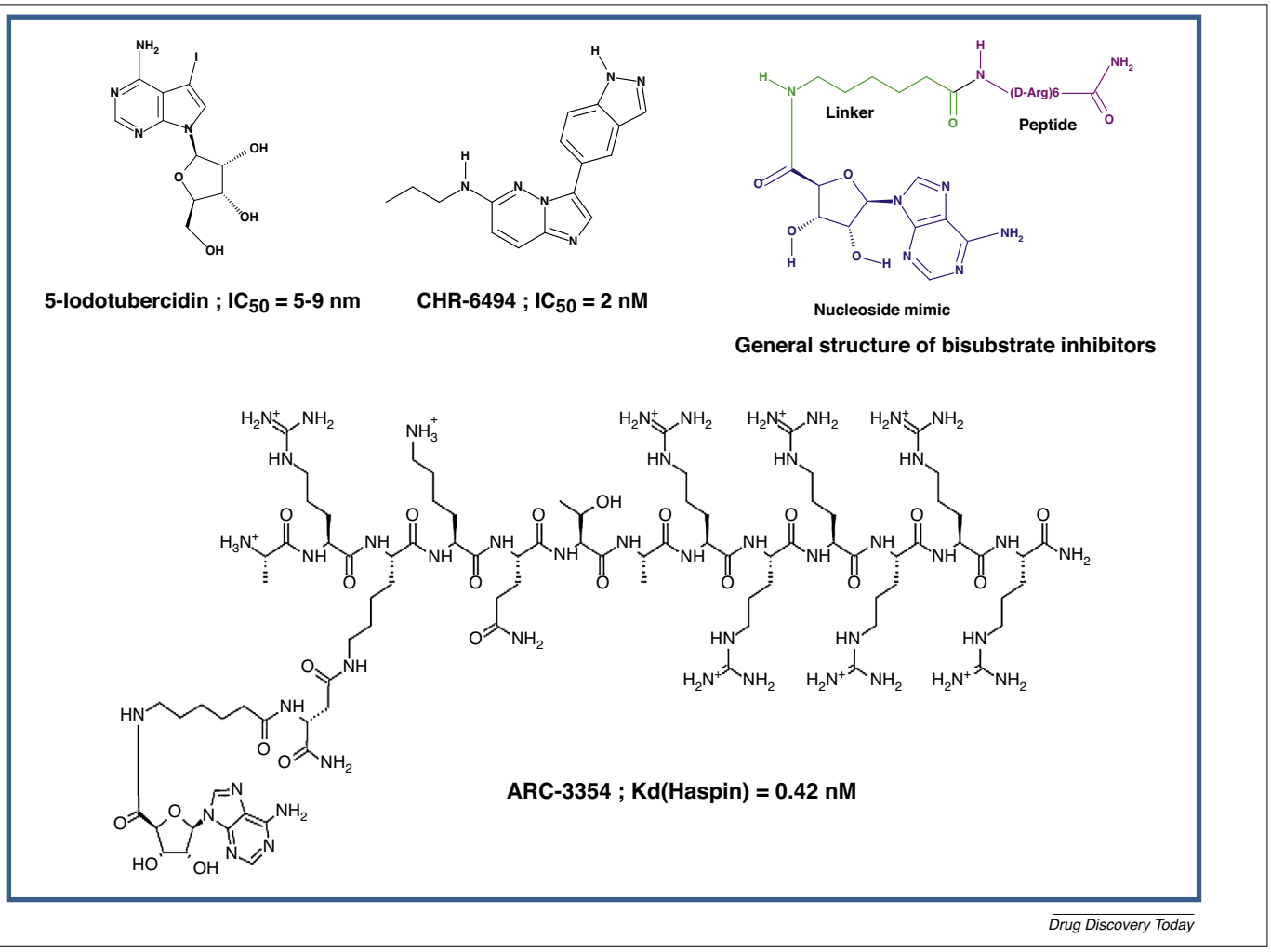


FIGURE 4

Chemical structures of other chemical families of haploid germ cell-specific nuclear protein kinase (Haspin) inhibitors.

TABLE 1

Targeting the cell cycle in cancer: an overview of validated and emerging strategies

Phase of cell cycle	Targets	
	Validated	**Potential**
Prophase	Cdk1, Aurora A, Plk1	Plk4, NEKs
Prometaphase	Microtubule-targeting agents, Cdk1, Aurora A, Aurora B, Plk1	Microtubule-associated Ser/Thr kinase-like, Human monopolar spindle (MPS1) kinase, Haspin
Metaphase	Microtubule-targeting agents, Aurora B	MPS1, Bub1, BubR1, NEKs, Haspin
Anaphase	N/A	Kinesin, Separase
Telophase/Cytokinesis	Aurora B, Plk1	Kinesin

dance with biological and clinical observations that inhibition of a small number of pertinent targets can be more efficient than the complete inhibition of a single target in cancer therapy. This could also lead to a new generation of anticancer compounds that reduces the number of co-administered drugs required per patient.

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