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3H-pyrazolo[4,3-f]quinoline haspin kinase inhibitors and anticancer properties

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Abstract

Histone modification, a post-translational modification of histones and involving various covalent tags, such as methyl, phosphate and acetate groups, affects gene expression and hence modulates various cellular events, including growth and proliferation. Consequently histone-modifying proteins have become targets for the development of anti-cancer agents. Thus far, compounds that inhibit the methylation or acetylation of histones have advanced in the clinic, but inhibitors of histone phosphorylation have lagged behind. Haspin is a kinase that phosphorylates histone H3 and is a promising anticancer target. Thus far only a handful of haspin inhibitors have been reported. Using a one flask Doebner/Povarov reaction, we synthesized a library of compounds that potently inhibit haspin with IC₅₀ values as low as 14 nM. Some of these compounds also inhibited the proliferation of cancer cell lines HCT116, HeLa and A375. The ease of synthesis of the new haspin inhibitors, coupled with their anticancer activities make these compounds interesting leads to develop into therapeutics.

Graphical abstract

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Conflict of Interest Statement

HOS is a co-founder of KinaRx LLC, a start-up company interested in developing oncology therapeutics.

Keywords

Haspin; Kinase inhibitor; Doebner/Povarov reaction

1. Introduction

Epigenetic mechanisms modulate heritable phenotypic changes that are not dictated by changes in DNA sequences. The link between epigenetic modifications and cancer development has been established[1, 2]. Histone modifications represent an important class of epigenetic mechanisms that could be targeted by drugs. The post-translational modifications of histones including methylation, acetylation, ubiquitination, phosphorylation and sumoylation regulate chromatin structure and affect the transcription of various genes[3]. As such intense efforts have been dedicated to the discovery and development of small molecule inhibitors of enzymes that control histone modifications as well as other epigenetic modification enzymes in various cancers[4–9].

Histone phosphorylation and de-phosphorylation is tightly regulated by epigenetic kinases and phosphatases[3, 10]. The phosphorylation of N-terminal tail of histone H3 at residues such as Thr3, S10, Thr11 and S28 has been well documented to regulate mitosis and meiosis in various life forms[3, 10]. Haspin (haploid germ cell-specific protein kinase), a serine/threonine kinase is involved in histone phosphorylation, particularly at Thr3 (H3T3) during mitosis[11, 12]. Haspin is expressed in several cell types and the upregulation or activation of haspin leads to H3T3 phosphorylation[3]. Dai *et al* demonstrated the vital role of haspin in chromosome alignment by showing that depletion of haspin via RNA interference resulted in decreased H3T3 phosphorylation, leading to the misalignment of chromosomes during metaphase[12]. Hence haspin is thought to play a role in chromosome segregation and might be important in tumor formation[11]. Despite the great potential of haspin being a target for anticancer therapy, only a handful of inhibitors have been documented[13–16]. CHR-6494, a haspin kinase inhibitor, was demonstrated to also possess antitumor properties against melanoma, colon, breast and cervical cancers[13, 17]. CHR-6494 caused a G₂/M cell cycle arrest consistent with the role of haspin in mitosis[11–13, 17].

Here we report that compounds containing the 3*H*-pyrazolo[4,3-*f*]quinoline scaffold potently inhibit haspin kinase. The compounds also possess anticancer activities against colon, cervical and skin cancers.

2. Results

2.1 Pyrazolo[4,3-*f*]quinoline compounds inhibit haspin kinase activity

We screened our in-house chemical library using the ADP-Glo™ kinase assay system (Promega, Madison WI) for haspin inhibitors and identified compound **1**, a phenolic compound that also contained a pyrazolo[4,3-*f*]quinoline moiety (synthesized via a Doebner/Povarov reaction[18–21], see Scheme 1), as haspin kinase inhibitors (see Figures 1 & 2).

For a structure-activity-relationship (SAR) study, we proceeded to synthesize a series of compound **1** analogs. In one series of analogs, we explored the effects of changing ring B

(see Scheme 1 for the naming of the rings) on haspin inhibition (compounds **2**, **3**, **4** and **5**). Next, we explored the effects of substituting position-1 with a methyl (compound **6**) or cyano (compound **7**) groups. For the third series of analogs, the phenolic moiety (ring A) was substituted with halogens (compounds **8**, **9**, **10** and **21**). We rationalized that the lower halogens (especially F) could affect the acidity of the phenolic OH without a confounding steric issue associated with higher halogen substitution (such as Br and I). The phenolic moiety was also changed to other aryl groups (such as phenyl (**14**), pyridine (**15**), hydroxypyridine (**12** and **13**), aniline (**16**), aminopyridine (**17**) and aminopyrimidine (**18**)). Finally, we designed two analogs to investigate the role that molecular architecture/geometry had on haspin inhibition. In other words, we designed analogs of compound **1**, which contained the same moieties but spatially arranged differently (compound **20** containing the 1*H*-pyrazolo[3,4-*f*]quinoline moiety, compound **19** containing 1*H*-pyrazolo[4,3-*h*]quinoline moiety to compare with compound **1**, which contains the 3*H*-pyrazolo[4,3-*f*]quinoline moiety). From the *in vitro* haspin inhibition assay, we observed varying degrees of activity from the compounds (Figure 2). Some compounds, such as **HSD972**, **HSD929**, and compound **17** were identified as potent inhibitors of haspin kinase (at 100 nM concentration, these three compounds inhibited over 90% of haspin's enzymatic activity) whilst other compounds, like compound **7**, had no inhibitory activity against haspin (Figure 2).

The SAR studies indicated that substitution at position 1 and the nature of ring B were important for haspin inhibition. For example, whereas the enzymatic inhibition profile of compound **6** (position-1 substituted with Me group) was similar to compound **1** (H at position 1), compound **7**, which had a cyano group at position 1 did not inhibit haspin (see Figure 2). The nature of halogen substitution on the phenolic moiety was also important for haspin inhibition. For example, both compounds **8** and **11** (**HSD972**) are monosubstituted with fluorine but compound **8**, which has the fluorine group *ortho* to the phenolic OH is less active against haspin than **HSD972**, which has the fluoro group *meta* to the phenolic OH (Figure 2). Additionally, we observed decreased potency with compounds **10** and **21**, which had two fluoro substitutions on ring A. Changing the phenolic OH into amino (compound **16**, **HSD929**) did not affect haspin inhibition. Replacement of the aniline moiety in compound **16** into aminopyridine (compound **17**) did not affect haspin inhibition. However, the pyrimidine analog, compound **18**, was slightly less active against haspin. Compounds **19** and **20** containing the 1*H*-pyrazolo[4,3-*h*]quinoline and 1*H*-pyrazolo[3,4-*f*]quinoline moieties were not as active against haspin than compound **1**, which contains the 3*H*-pyrazolo[4,3-*f*]quinoline moiety (Figure 2). Therefore it appears that the 3*H*-pyrazolo[4,3-*f*]quinoline moiety is the most optimum core scaffold for developing this class of haspin inhibitors.

From the single concentration inhibition data (Figure 2), compounds **HSD972**, **HSD929** and **17** were chosen for more detailed investigation of haspin inhibition. We determined the half maximal inhibitory concentration (IC₅₀) of these top three active compounds against haspin (Figure 3). From the IC₅₀ analysis, we observed that **HSD972** was most potent against haspin with an IC₅₀ value of 14 nM, compared with 45 nM and 61 nM for compounds **17** and **HSD929** respectively (Figure 3).

2.2 Antiproliferative activities of compounds

As earlier stated, haspin inhibitors have been demonstrated to have anticancer activities against various cancers[13, 17]. Following the impressive haspin inhibition activities of the compounds, particularly **HSD972**, **HSD929** and compound **17**, we proceeded to investigate whether these compounds possessed any anticancer activities. Precedent literature demonstrated the anticancer activity of CHR-6494 (a haspin inhibitor) against the colon cancer cell line HCT116, the cervical cancer cell line HeLa and as well as human melanoma cell lines RPMI-7951 and COLO-792[13, 17]. We determined the anticancer activities of the synthesized compounds (Figure 1) against, HCT116, HeLa and A375 cell lines by incubating the cell lines with 10 μ M of the compounds and determining growth inhibition after 72 h. The compounds displayed varied potencies against the selected cancer cell lines. HCT116 and HeLa cell lines were more sensitive to the compounds than the melanoma A375 cell line (Figure 4). There appeared to be some correlation between haspin inhibition and antiproliferative activity of the most active compounds. For example, compounds **7** and **19**, which were poor haspin inhibitors performed poorly in the cellular assays. Compound **12** on the other hand, was a poor haspin inhibitor yet it displayed a moderate antiproliferative activity against HCT116, HeLa and A375 cancer cell lines. This suggests that compound **12** inhibits other targets and future studies will attempt to identify the true target of **12**. **HSD929** and **HSD972** were both potent inhibitors of haspin and possessed potent antiproliferative activities against HCT116 and HeLa cell lines (Figure 4). An outlier was compound **1**, which is a good haspin inhibitor but did not have good anticancer activities against the three tested cell lines. Various factors, such as drug permeation, stability etc. affect phenotypic outcomes and one of such factors could account for the low correlation between the high degree of haspin inhibition by compound **1** and anticancer activities.

Since compounds **HSD929** and **HSD972** displayed the highest growth inhibition at 10 μ M, we decided to determine the half maximum inhibitory concentration (IC₅₀) against HCT116 and HeLa cell lines (Figure 5). **HSD929** inhibited HCT116 and HeLa cell lines in a dose-dependent manner with IC₅₀ values of 1.68 μ M and 3.6 μ M respectively. **HSD972** also had similar potency as **HSD972** with IC₅₀ values of 2.7 μ M and 4.8 μ M against both cell lines.

2.3 Haspin-HSD972 interaction at hinge region is similar to haspin-ADP interaction

To gain insights into the interaction between the active compounds and haspin, we used GLIDE in the Schrodinger software package to dock **HSD972** to haspin (PDB 3DLZ)[22]. Docking results indicated that **HSD972** docked to the active site (Figure 6). **HSD972** formed a hydrogen bond interaction with the backbone carbonyl oxygen of Glu606, a hinge region residue[22].

The haspin Glu606 residue is known to interact with ATP in the binding pocket[22]. The N6-amino group of ADP purine ring makes a hydrogen bond contact with Glu606[22]. Interestingly, when the structures of **HSD972** and crystalized ADP molecule were superimposed, the N6-amino group of the purine ring of ADP overlaid with the N3 atom of the 8,9,10,11-tetrahydro-3H-pyrazolo[4,3-a]phenanthridine moiety of **HSD972** (Figure 6). Both nitrogen atoms are attached to hydrogens that could serve as hydrogen bond donors to the backbone carbonyl oxygen of Glu606. Electrostatic surface potential map analysis of

HSD972 revealed that the N3-H moiety had a partial positive charge (blue color, Figure S1), which allows it to be a hydrogen bond donor. Analysis of the electrostatic surface potential of haspin reveals hydrophobic patches (δ°) (Figure 6) in the active site created by residues such as Phe495, Val498 and Ile686. The hydrophobic cyclohexyl ring B of **HSD972** docks in this pocket (Figure 6 C and D).

2.4 HSD972 affects cell cycle progression and apoptosis

The haspin inhibitor CHR-6494 was shown to induce a G₂/M cell cycle arrest as well as apoptosis in HCT116 and HeLa cells[13]. Therefore, we wondered whether **HSD972** could also induce cell cycle arrest and apoptosis in HCT116 cell line. Using the propidium iodide (PI)-staining with flow cytometry, we determined the distribution of cells in the different cell cycle phases after 24 h of treatment with **HSD972** (5X IC₅₀). It was observed that compared to the DMSO-treated cells, the population of cells in the G₂/M phase was higher in the presence of **HSD972** (Figure 7). A corresponding decrease in the population of cells in G₁ phase was observed. A caspase 3/7 activity assay also revealed that **HSD972** increased the activity of caspase 3/7 (a sign of apoptosis) after 72 hours in HCT116 cells, see Figure 8.

3. Conclusions

In this report we have identified inhibitors of haspin kinase activity and demonstrated their ability to inhibit cancer cell proliferation. **HSD972** and **HSD929** were the most potent inhibitors with nanomolar IC₅₀ values for the inhibition of haspin activity. Both compounds also inhibited HCT116 and HeLa proliferation at single digit micromolar concentrations. The ease of synthesis of the reported compounds (a one flask synthesis) makes these novel pyrazolo[4,3-*f*]quinoline-containing kinase inhibitors ideal lead compounds to develop into anticancer therapeutics.

4. Experimental

4.1 Chemistry

4.1.1 General considerations—Unless noted otherwise, all reagents and solvents were purchased from commercial sources and used as received. All reactions were performed in a screw-cap 20 mL vials. The ¹H and ¹³C NMR spectra were obtained in CD₃OD or (CD₃)₂SO as solvent using a 500 MHz spectrometer with an internal standard Me₄Si. Chemical shifts are reported in parts per million (δ) and are calibrated using residual undeuterated solvent as an internal reference. Data for ¹H NMR spectra are reported as follows: chemical shift (δ ppm) (multiplicity, coupling constant (Hz), integration). Multiplicities are reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, or combinations thereof. High resolution mass spectra (HRMS) were given using electron spray ionization (ESI) technique and as TOF mass analyzer. All the synthesized compounds were characterized by ¹H NMR, ¹³C NMR, and HRMS data.

4.1.2 General procedure for the multicomponent reaction—A mixture of amine (1 mmol) and aldehyde (1 mmol) in 3 mL of absolute ethanol was refluxed for 2 h followed by addition of cyclic ketone (2.5 mmol) to the reaction mixture. A catalytic amount of conc.

hydrochloric acid was added, and the reaction was continued to reflux for 6–12 h. Reaction mixture dissolved in ethyl acetate (50 mL), washed with sodium bicarbonate solution and water (10 mL × 2). The organic layer was dried (Na₂SO₄), concentrated under reduced pressure, and purified by silica gel chromatography [dichloromethane:methanol (99:01 to 80:20) or hexane:ethylacetate (20:80 to 05:95)] to give the desired cyclized compound. (Note: Sometime product may get precipitated out, which was filtered, washed with absolute ethanol and further purified by column chromatography).

4.1.2.1 4-(8,9,10,11-Tetrahydro-3H-pyrazolo[4,3-*a*]phenanthridin-7-yl)phenol (1): Off white solid (189 mg, 60%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.66 (s, 1H), 8.54 (s, 1H), 7.83 (q, *J* = 9.1 Hz, 2H), 7.48 – 7.29 (m, 2H), 7.02 – 6.73 (m, 2H), 3.33–3.34 (m, 2H), 2.79 (t, *J* = 6.1 Hz, 2H), 2.13 – 1.94 (m, 2H), 1.85 – 1.58 (m, 2H); ¹³C NMR (126 MHz, DMSO) δ 157.71, 156.82, 143.40, 138.61, 136.25, 131.76, 130.89, 129.56, 121.53, 116.48, 116.04, 115.15, 114.37, 29.76, 29.18, 22.66, 22.63; HRMS (ESI) *m/z* calcd for C₂₀H₁₈N₃O [M + H]⁺ 316.1449, found 316.1458.

4.1.2.2 4-(8,9,10,11-Tetrahydro-3H-8,11-methanopyrazolo[4,3-*a*]phenanthridin-7-yl)phenol (2): A mixture of indazole (1 mmol), 4-hydroxybenzaldehyde (1 mmol) and norcamphor (2 mmol) in 6 mL tetrahydrofuran was refluxed for 6–12 h in presence of iodine (10 mol%). After completion of reaction, reaction mixture was extracted with ethyl acetate and washed with sodium thiosulphate aqueous solution (5%). The organic layer was dried (Na₂SO₄), concentrated under reduced pressure, purified by silica gel chromatography [ethyl acetate:hexane (60:40)] to give the desired cyclized compound.

Off white solid (82 mg, 25%). ¹H NMR (500 MHz, MeOD-*d*₄) δ 8.64 (s, 1H), 7.97 – 7.90 (m, 1H), 7.80 (d, *J* = 9.2 Hz, 1H), 7.62 (d, *J* = 8.4 Hz, 2H), 6.97 (d, *J* = 8.4 Hz, 2H), 4.34 (s, 1H), 3.76 (s, 1H), 2.33 – 2.18 (m, 2H), 1.93 (d, *J* = 9.4 Hz, 1H), 1.77 (d, *J* = 9.0 Hz, 1H), 1.44 – 1.31 (m, 2H). ¹³C NMR (126 MHz, MeOD) δ 157.96, 153.18, 150.46, 144.56, 139.98, 138.03, 134.29, 130.56, 130.03, 128.51, 116.16, 114.92, 113.81, 49.47, 43.72, 42.28, 26.38, 24.64. HRMS (ESI) *m/z* calcd for C₂₁H₁₈N₃O [M + H]⁺ 328.1450, found 328.1459.

4.1.2.3 4-(3,8,9,10-Tetrahydrocyclopenta[*c*]pyrazolo[4,3-*f*]quinolin-7-yl)phenol (3): Off-white solid (168 mg, 56%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.42 (s, 1H), 7.85 (d, *J* = 9.2 Hz, 1H), 7.81 (dd, *J* = 9.1, 0.9 Hz, 1H), 7.74 (d, *J* = 8.6 Hz, 2H), 6.89 (d, *J* = 8.6 Hz, 2H), 3.41 (t, *J* = 7.6 Hz, 2H), 3.21 (t, *J* = 7.4 Hz, 2H), 2.20 – 2.22 (m, 2H); ¹³C NMR (126 MHz, DMSO) δ 158.20, 152.26, 149.32, 144.91, 135.59, 131.44, 130.38, 129.22, 118.73, 116.70, 115.46, 33.48, 33.40, 24.97; HRMS (ESI) *m/z* calcd for C₁₉H₁₆N₃O [M + H]⁺ 302.1293, found 302.1295.

4.1.2.4 4-(8,9-Dihydro-3H-cyclobuta[*c*]pyrazolo[4,3-*f*]quinolin-7-yl)phenol (4): Off-white solid (152 mg, 53%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.80 (s, 1H), 8.32 (s, 1H), 8.01 (dd, *J* = 8.6, 1.4 Hz, 2H), 7.82 (d, *J* = 9.5 Hz, 1H), 7.78 (d, *J* = 9.1 Hz, 1H), 6.91 (dd, *J* = 8.6, 1.4 Hz, 2H), 3.73 – 3.64 (m, 2H), 3.62 – 3.54 (m, 2H); ¹³C NMR (126 MHz, DMSO) δ 159.12, 149.75, 148.04, 144.97, 137.96, 136.56, 134.42, 130.02, 129.03, 128.87, 116.88,

116.11, 115.24, 114.32, 32.33, 30.24; HRMS (ESI) m/z calcd for $C_{18}H_{14}N_3O$ $[M + H]^+$ 288.1137, found 288.1139.

4.1.2.5 4-(3,8,10,11-Tetrahydropyrano[3,4-c]pyrazolo[4,3-f]quinolin-7-yl)phenol

(5): Off-white solid (206 mg, 65%). 1H NMR (500 MHz, DMSO- d_6) δ 9.69 (s, 1H), 8.52 (s, 1H), 7.85 (q, J = 9.2 Hz, 2H), 7.40 (d, J = 8.5 Hz, 2H), 6.87 (d, J = 8.6 Hz, 2H), 4.77 (s, 2H), 4.11 (t, J = 5.9 Hz, 2H), 3.34 (d, J = 6.8 Hz, 2H); ^{13}C NMR (126 MHz, DMSO) δ 158.09, 153.88, 144.20, 139.12, 138.55, 135.81, 130.66, 130.59, 129.53, 127.54, 120.80, 116.34, 115.40, 114.82, 67.17, 64.45, 28.70; HRMS (ESI) m/z calcd for $C_{19}H_{16}N_3O_2$ $[M + H]^+$ 318.1243, found 318.1244.

4.1.2.6 4-(1-Methyl-8,9,10,11-tetrahydro-3H-pyrazolo[4,3-a]phenanthridin-7-yl)phenol

(6): Off-white solid (132 mg, 40%). 1H NMR (500 MHz, DMSO- d_6) δ 10.37 (s, 1H), 8.24 – 8.03 (m, 2H), 7.56 (d, J = 8.6 Hz, 2H), 7.04 (d, J = 8.6 Hz, 2H), 3.62 (t, J = 5.9 Hz, 2H), 2.94 (s, 3H), 2.81 (t, J = 6.2 Hz, 2H), 1.93 – 1.73 (m, 4H); ^{13}C NMR (126 MHz, DMSO) δ 163.50, 160.27, 153.31, 150.55, 135.81, 135.11, 131.86, 131.49, 125.28, 122.45, 119.96, 116.00, 112.81, 32.79, 27.62, 21.69, 21.32; HRMS (ESI) m/z calcd for $C_{21}H_{20}N_3O$ $[M + H]^+$ 330.1606, found 330.1606.

4.1.2.7 7-(4-Hydroxyphenyl)-8,9,10,11-tetrahydro-3H-pyrazolo[4,3-a]phenanthridine-1-carbonitrile (7):

White solid (210 mg, 61%). 1H NMR (500 MHz, MeOD- d_4) δ 8.32 (d, J = 9.3 Hz, 1H), 8.20 (d, J = 9.2 Hz, 1H), 7.62 (d, J = 8.6 Hz, 2H), 7.09 (d, J = 8.5 Hz, 2H), 3.91 (t, J = 6.3 Hz, 2H), 3.00 (t, J = 6.3 Hz, 2H), 2.24 – 2.09 (m, 2H), 2.01 – 1.90 (m, 2H). ^{13}C NMR (126 MHz, MeOD) δ 160.62, 155.55, 153.29, 139.97, 135.54, 133.16, 130.98, 122.63, 121.71, 121.08, 119.18, 115.88, 115.65, 115.28, 33.30, 27.88, 20.92, 20.63; HRMS (ESI) m/z calcd for $C_{21}H_{21}N_4O$ $[M + H]^+$ 345.1715, found 345.1720.

4.1.2.8 2-Fluoro-4-(8,9,10,11-tetrahydro-3H-pyrazolo[4,3-a]phenanthridin-7-yl)phenol

(8): Off white solid (207 mg, 62%). 1H NMR (500 MHz, DMSO- d_6) δ 10.81 (s, 1H), 8.83 (s, 1H), 8.26 (d, J = 9.2 Hz, 1H), 8.21 (d, J = 9.2 Hz, 1H), 7.66 (dd, J = 11.8, 2.2 Hz, 1H), 7.42 (dd, J = 8.3, 2.1 Hz, 1H), 7.26 (t, J = 8.6 Hz, 1H), 3.49 (t, J = 6.5 Hz, 2H), 2.83 (t, J = 6.2 Hz, 2H), 2.11 – 1.98 (m, 2H), 1.84 – 1.70 (m, 2H); ^{13}C NMR (126 MHz, DMSO) δ 154.99, 151.91, 149.98, 147.90, 135.34, 132.04, 127.34, 123.18, 120.34, 118.67, 118.51, 118.31, 114.88, 40.49, 40.33, 40.25, 40.16, 40.08, 39.99, 39.83, 39.66, 39.49, 30.93, 28.11, 21.80, 21.69; HRMS (ESI) m/z calcd for $C_{20}H_{17}FN_3O$ $[M + H]^+$ 334.1355, found 334.1351.

4.1.2.9 2-Chloro-4-(8,9,10,11-tetrahydro-3H-pyrazolo[4,3-a]phenanthridin-7-yl)phenol

(9): Off-white solid (191 mg, 55%). 1H NMR (500 MHz, DMSO- d_6) δ 11.19 (s, 1H), 8.68 (s, 1H), 8.14 (s, 2H), 7.78 (d, J = 2.2 Hz, 1H), 7.56 (dd, J = 8.4, 2.2 Hz, 1H), 7.31 (d, J = 8.5 Hz, 1H), 3.33 (d, J = 6.6 Hz, 2H), 2.80 (d, J = 6.3 Hz, 2H), 2.00 (dt, J = 12.4, 4.6 Hz, 2H), 1.80 – 1.69 (m, 2H); ^{13}C NMR (126 MHz, DMSO) δ 155.92, 152.58, 149.60, 135.00, 131.94, 131.83, 130.50, 123.67, 122.81, 120.32, 120.19, 116.98, 114.53, 30.78, 28.06, 21.65, 21.61; HRMS (ESI) m/z calcd for $C_{20}H_{17}ClN_3O$ $[M + H]^+$ 350.1060, found 350.1066.

4.1.2.10 2,5-Difluoro-4-(8,9,10,11-tetrahydro-3H-pyrazolo[4,3-*a*]phenanthridin-7-

yl)phenol (10): Off-white solid (159 mg, 45%). ¹H NMR (500 MHz, Methanol-*d*₄) δ 8.57 (s, 1H), 7.86 (q, *J* = 9.1 Hz, 2H), 7.15 (dd, *J* = 10.8, 6.7 Hz, 1H), 6.81 (dd, *J* = 10.5, 7.1 Hz, 1H), 3.38 (s, 2H), 2.73 (s, 2H), 2.06 – 2.09 (m, 2H), 1.86 (d, *J* = 9.8 Hz, 2H); ¹³C NMR (126 MHz, MeOD) δ 156.74 (*J* = 239.16 Hz), 151.59, 148.92 (*J* = 238.14 Hz), 146.61, 143.45, 143.06, 138.67, 135.89, 130.94, 128.32, 122.39, 118.20, 116.93, 116.06, 113.85, 104.69, 29.48, 27.05, 22.11, 21.80; HRMS (ESI) *m/z* calcd for C₂₀H₂₁F₂N₃O [M + H]⁺ 354.1418, found 354.1421.

4.1.2.11 3-Fluoro-4-(8,9,10,11-tetrahydro-3H-pyrazolo[4,3-*a*]phenanthridin-7-yl)phenol

(11, HSD972): White solid (186 mg, 56%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.57 (s, 1H), 7.84 (dd, *J* = 9.0, 0.9 Hz, 1H), 7.79 (d, *J* = 9.1 Hz, 1H), 7.22 (t, *J* = 8.6 Hz, 1H), 6.72 (dd, *J* = 8.3, 2.3 Hz, 1H), 6.66 (dd, *J* = 11.8, 2.3 Hz, 1H), 3.30 (t, *J* = 6.6 Hz, 2H), 2.61 (t, *J* = 6.0 Hz, 2H), 2.00 – 1.91 (m, 2H), 1.79 – 1.69 (m, 2H); ¹³C NMR (126 MHz, DMSO) δ 161.25 (*J* = 245.11 Hz), 159.59, 152.73, 143.80, 142.04, 132.20, 132.16, 130.53, 129.53, 122.03, 119.48, 119.35, 116.25, 112.08, 102.89, 102.70, 29.48, 27.50, 22.64, 22.24; HRMS (ESI) *m/z* calcd for C₂₀H₁₇FN₃O [M + H]⁺ 334.1356, found 334.1360.

4.1.2.12 5-(8,9,10,11-Tetrahydro-3H-pyrazolo[4,3-*a*]phenanthridin-7-yl)pyridin-2-ol

(12): ¹H NMR (500 MHz, DMSO-*d*₆) δ 11.86 (s, 1H), 8.53 (s, 1H), 7.83 (d, *J* = 9.0 Hz, 1H), 7.80 – 7.73 (m, 2H), 7.65 (d, *J* = 2.6 Hz, 1H), 6.42 (d, *J* = 9.4 Hz, 1H), 3.25 (t, *J* = 6.6 Hz, 2H), 2.84 (t, *J* = 6.1 Hz, 2H), 2.01 – 1.92 (m, 2H), 1.80 – 1.68 (m, 2H); ¹³C NMR (126 MHz, DMSO) δ 162.21, 152.68, 143.74, 142.84, 142.59, 136.37, 129.51, 129.43, 121.76, 119.38, 118.73, 116.21, 29.69, 28.78, 22.59, 22.54; HRMS (ESI) *m/z* calcd for C₁₉H₁₇N₄O [M + H]⁺ 317.1402, found 317.1411.

4.1.2.13 6-(8,9,10,11-Tetrahydro-3H-pyrazolo[4,3-*a*]phenanthridin-7-yl)pyridin-3-ol

(13): Off-white solid (124 mg, 39%). ¹H NMR (500 MHz, MeOD-*d*₄) δ 8.59 (s, 1H), 8.22 (dd, *J* = 2.8, 0.7 Hz, 1H), 7.91 (d, *J* = 9.7 Hz, 1H), 7.84 (d, *J* = 9.0 Hz, 1H), 7.56 (dd, *J* = 8.4, 0.7 Hz, 1H), 7.41 (dd, *J* = 8.5, 2.8 Hz, 1H), 3.46 – 3.35 (m, 2H), 2.83 (t, *J* = 6.2 Hz, 2H), 2.12 – 2.04 (m, 2H), 1.90 – 1.79 (m, 2H); ¹³C NMR (126 MHz, MeOD) δ 154.91, 154.11, 149.18, 143.77, 142.83, 138.73, 136.11, 135.92, 130.22, 128.53, 125.24, 123.34, 122.50, 116.13, 113.82, 29.64, 27.64, 22.11, 21.92; HRMS (ESI) *m/z* calcd for C₁₉H₁₇N₄O [M + H]⁺ 317.1402, found 317.1397.

4.1.2.14 7-Phenyl-8,9,10,11-tetrahydro-3H-pyrazolo[4,3-*a*]phenanthridine (14):

Off-white solid (235 mg, 78%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.56 (s, 1H), 7.84 (dd, *J* = 9.1, 0.9 Hz, 1H), 7.80 (d, *J* = 9.1 Hz, 1H), 7.54 – 7.51 (m, 2H), 7.48 – 7.44 (m, 2H), 7.44 – 7.39 (m, 1H), 3.29 (t, *J* = 6.5 Hz, 2H), 2.74 (t, *J* = 6.1 Hz, 2H), 2.02 – 1.91 (m, 2H), 1.77 – 1.64 (m, 2H); ¹³C NMR (126 MHz, DMSO) δ 156.89, 143.73, 142.34, 141.33, 139.70, 135.12, 129.62, 129.45, 129.22, 128.39, 128.15, 121.89, 116.26, 115.24, 29.64, 28.95, 22.59, 22.49; HRMS (ESI) *m/z* calcd for C₂₀H₁₈N₃ [M + H]⁺ 300.1501, found 300.1501.

4.1.2.15 7-(Pyridin-3-yl)-8,9,10,11-tetrahydro-3H-pyrazolo[4,3-*a*]phenanthridine

(15): Off-white solid (135 mg, 45%). ¹H NMR (500 MHz, MeOD-*d*₄) δ 9.06 (s, 2H), 8.73

(s, 1H), 8.42 (d, $J = 7.3$ Hz, 1H), 8.11 (d, $J = 8.4$ Hz, 1H), 7.96 (d, $J = 8.8$ Hz, 2H), 3.55 – 3.46 (m, 2H), 2.95 – 2.81 (m, 2H), 2.20 – 2.08 (m, 2H), 1.99 – 1.84 (m, 2H); ^{13}C NMR (126 MHz, MeOD) δ 150.86, 148.85, 148.27, 147.36, 140.15, 138.36, 131.44, 123.81, 122.94, 119.15, 115.51, 30.47, 27.89, 21.51, 21.42; HRMS (ESI) m/z calcd for $\text{C}_{19}\text{H}_{17}\text{N}_4$ $[\text{M} + \text{H}]^+ 301.1453$, found 301.1465.

4.1.2.16 4-(8,9,10,11-Tetrahydro-3H-pyrazolo[4,3-a]phenanthridin-7-yl)aniline

(16): Orange solid (192 mg, 61%). ^1H NMR (500 MHz, DMSO- d_6) δ 8.60 (s, 1H), 8.03 – 7.90 (m, 2H), 7.38 (d, $J = 8.2$ Hz, 2H), 6.74 (d, $J = 8.2$ Hz, 2H), 3.30 (t, $J = 6.4$ Hz, 2H), 2.85 – 2.77 (m, 2H), 2.05 – 1.89 (m, 2H), 1.79 – 1.65 (m, 2H); ^{13}C NMR (126 MHz, DMSO) δ 162.76, 154.32, 150.10, 147.41, 138.78, 136.07, 131.27, 130.64, 124.43, 121.78, 115.78, 114.97, 113.81, 111.39, 30.33, 28.84, 22.22; HRMS (ESI) m/z calcd for $\text{C}_{20}\text{H}_{19}\text{N}_4$ $[\text{M} + \text{H}]^+ 315.1610$, found 315.1612.

4.1.2.17 5-(8,9,10,11-Tetrahydro-3H-pyrazolo[4,3-a]phenanthridin-7-yl)pyridin-2-

amine (17): Yellow solid (205 mg, 65%). ^1H NMR (500 MHz, MeOD- d_4) δ 8.51 (s, 1H), 8.16 (d, $J = 2.1$ Hz, 1H), 8.14 (dd, $J = 9.0, 2.2$ Hz, 1H), 7.88 (d, $J = 9.1$ Hz, 1H), 7.83 (d, $J = 9.2$ Hz, 1H), 7.09 (d, $J = 9.0$ Hz, 1H), 3.26 (t, $J = 6.6$ Hz, 2H), 2.87 (t, $J = 6.1$ Hz, 2H), 2.06 (ddt, $J = 9.2, 6.5, 3.2$ Hz, 2H), 1.87 – 1.84 (m, 2H); ^{13}C NMR (126 MHz, MeOD) δ 155.32, 150.42, 145.84, 143.67, 143.48, 141.93, 138.13, 130.19, 126.90, 123.86, 122.70, 119.27, 115.60, 111.95, 29.80, 28.11, 21.86, 21.86; HRMS (ESI) m/z calcd for $\text{C}_{19}\text{H}_{18}\text{N}_5$ $[\text{M} + \text{H}]^+ 316.1562$, found 316.1559.

4.1.2.18 5-(8,9,10,11-Tetrahydro-3H-pyrazolo[4,3-a]phenanthridin-7-yl)pyrimidin-2-

amine (18): Yellow solid (152 mg, 48%); ^1H NMR (500 MHz, DMSO- d_6) δ 8.61 – 8.47 (m, 3H), 7.84 (s, 2H), 6.85 (s, 2H), 3.35 – 3.30 (m, 2H), 2.94 – 2.85 (m, 2H), 2.04 – 1.95 (m, 2H), 1.83 – 1.73 (m, 2H); ^{13}C NMR (126 MHz, DMSO) δ 163.24, 158.64, 152.40, 143.92, 142.53, 138.65, 136.38, 129.80, 129.66, 123.40, 121.74, 116.45, 114.46, 29.74, 28.82, 22.64, 22.59; HRMS (ESI) m/z calcd for $\text{C}_{18}\text{H}_{17}\text{N}_6$ $[\text{M} + \text{H}]^+ 317.1515$, found 317.1515.

4.1.2.19 4-(6,7,8,9-Tetrahydro-3H-pyrazolo[4,3-c]phenanthridin-5-yl)phenol (19): Pale yellow solid (161 mg, 51%). ^1H NMR (500 MHz, MeOD- d_4) δ 8.10 (s, 1H), 7.73 (d, $J = 9.0$ Hz, 1H), 7.59 (d, $J = 9.0$ Hz, 1H), 7.38 (d, $J = 8.5$ Hz, 2H), 6.92 (d, $J = 8.6$ Hz, 2H), 3.18 (t, $J = 6.5$ Hz, 2H), 2.76 (t, $J = 6.2$ Hz, 2H), 2.03 – 1.91 (m, 2H), 1.80 – 1.70 (m, 2H); ^{13}C NMR (126 MHz, MeOD) δ 159.00, 157.94, 143.72, 137.36, 134.24, 133.58, 131.34, 130.05, 130.02, 129.40, 125.19, 121.58, 119.04, 116.10, 114.76, 28.52, 26.20, 22.31, 21.98; HRMS (ESI) m/z calcd for $\text{C}_{20}\text{H}_{18}\text{N}_3\text{O}$ $[\text{M} + \text{H}]^+ 316.1450$, found 316.1459.

4.1.2.20 4-(8,9,10,11-Tetrahydro-1H-pyrazolo[3,4-a]phenanthridin-7-yl)phenol

(20): Off-white solid (182 mg, 58%). ^1H NMR (500 MHz, DMSO- d_6) δ 8.31 (s, 1H), 7.87 (d, $J = 8.9$ Hz, 1H), 7.51 (d, $J = 8.9$ Hz, 1H), 7.39 (d, $J = 8.5$ Hz, 2H), 6.85 (d, $J = 8.5$ Hz, 2H), 3.50 (t, $J = 6.6$ Hz, 2H), 2.79 (t, $J = 6.1$ Hz, 2H), 1.97 – 1.92 (m, 2H), 1.77 – 1.70 (m, 2H); ^{13}C NMR (126 MHz, DMSO) δ 158.40, 157.95, 146.70, 142.55, 131.73, 130.87, 128.50, 124.50, 122.19, 119.87, 116.93, 115.17, 29.97, 29.12, 22.56; HRMS (ESI) m/z calcd for $\text{C}_{20}\text{H}_{18}\text{N}_3\text{O}$ $[\text{M} + \text{H}]^+ 316.1450$, found 316.1443.

4.1.2.21 2,3-Difluoro-4-(8,9,10,11-tetrahydro-3H-pyrazolo[4,3-a]phenanthridin-7-

yl)phenol (21): Off-white solid (177 mg, 50%). ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.56 (s, 1H), 8.56 (s, 1H), 7.92 – 7.76 (m, 2H), 7.05 (td, *J* = 8.2, 2.0 Hz, 1H), 6.90 (td, *J* = 8.3, 1.6 Hz, 1H), 3.33–3.32 (m, 2H), 2.62 (t, *J* = 6.1 Hz, 2H), 1.96 (dt, *J* = 11.1, 5.9 Hz, 2H), 1.75 – 1.73 (m, 2H); ¹³C NMR (126 MHz, DMSO) δ 151.48, 149.77(d, *J* = 243.18 Hz), 147.03, 143.67, 142.40, 141.06 d, *J* = 240.66 Hz), 138.74, 136.40, 130.47, 129.50, 125.40, 122.18, 120.81, 116.39, 114.56, 113.32, 29.50, 27.48, 22.58, 22.19; HRMS (ESI) *m/z* calcd for C₂₀H₁₆F₂N₃O [M + H]⁺ 352.1261, found 352.1256.

4.2 Biological Evaluation

4.2.1 Materials—Haspin kinase was purchased from SignalChem (Richmond, BC, Canada). ADP-Glo™ kinase assay system and CellTiter-Blue® cell viability assay were purchased from Promega (Madison, WI, USA).

4.2.2 Cells and cell culture—HCT116, HeLa and A375 cells were used in this study. Routinely, HCT116 was cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS). HeLa and A375 cells were cultured in DMEM medium supplemented with 10% FBS. Cultures were incubated at 37 °C with 5% CO₂.

4.2.3 Haspin kinase activity—The effect of compounds on haspin kinase activity was assayed using the ADP-Glo™ kinase assay system (Promega, Madison WI). The kinase reaction was performed in 384 well plates in a 5 µL volume containing reaction buffer (25 mM MOPS (pH 7.2) 12.5mM β-glycerol-phosphate, 25 mM MgCl₂, 5 mM EGTA, 2 mM EDTA, 0.25 mM DTT and 0.1 mg/ml BSA), 10 µM ATP, 0.1 mg/mL casein protein with 100 nM compound and 5 ng/µL haspin kinase. The reaction was incubated for 3 h at room temperature (23 – 25 °C) after which the ADP-Glo™ kinase assay system was used to determine haspin activity following the manufacturer's protocol.

4.2.4 Antiproliferative activity—To determine the effect of compounds on the proliferation of cancer cells, HCT116, HeLa and A375 cells were seeded into 96 well plates at 4000 cells/well for HCT116 and 2000 cells/well for HeLa and A375 cells in 190 µL of medium. After 18 h to 24 h, cultures were treated with 10 µL of compounds diluted in medium to yield a final concentration of 10 µM for the screening. For the dose response curves, compounds were serially diluted in DMSO before being further diluted into medium. The cultures were ended after 72 h by the addition of 10 µL CellTiter-Blue® cell viability assay (Promega, Madison WI). The plates were incubated for a further 4 hours before the viability assayed by measuring the fluorescence with excitation at 560 nm and emission at 590 nm on a BioTek Cytation 5 Cell Imaging multi-mode reader. Data were normalized to the DMSO control. Dose-response curves were generated by fitting data to a non-linear regression analysis using GraphPad Prism 5.0 Software (La Jolla CA).

4.2.5 Molecular docking analysis—Docking of **HSD972** to haspin (PDB 3DLZ) [22] was performed using the Schrodinger software packages using default settings. Briefly, **HSD972** was optimized for docking using the default settings of the LigPrep module. Haspin (PDB 3DLZ) was imported into Maestro 11.3.016 and the Protein preparation

wizard was used to optimize the PDB file. The Receptor Grid Generation module was used to generate a grid around the active site, which was used in the Ligand docking module with the optimized ligands for docking. **HSD972** and ADP were both viewed in the active site of Haspin at the same time to determine the binding position of **HSD972** relative to ADP.

4.2.6 Cell cycle analysis—Cell cycle analysis was performed using propidium iodide as a nuclear stain. HCT116 cells (2×10^6) were seeded and incubated with **HSD972** for overnight. Harvested cells were fixed with 70% ethanol following overnight incubation at $-20\text{ }^{\circ}\text{C}$. In the next step, the cells were treated with propidium iodide and RNase and analyzed by flow cytometry (BD Bioscience).

4.2.7 Apoptosis—Caspase 3/7 assay was carried out using Caspase 3/7 fluorescence assay kit (Cayman Chemicals). Briefly, 5×10^5 HCT116 cells were seeded in 12 well plates. The cells were treated with HSD972 and were collected after 24, 48 and 72 hours. Fluorescence intensity was measured by BioTek Cytation 5 plate reader.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- One flask synthesis of haspin kinase inhibitors via Doebner/Povarov reaction.
- Most active inhibitor, **HSD972**, inhibits haspin with IC₅₀ of 14 nM.
- Novel pyrazolo[4,3-*f*]quinoline-containing haspin inhibitors could inhibit the proliferation of HCT116, Hela and A375 cell lines.
- **HSD972** induces a G₂/M cell cycle arrest.
- **HSD972** causes an increase in caspase 3/7 activities in HCT 116 cells.

Figure 1.
Structures of **HSD972** and related compounds

Figure 2.

The effect of **HSD972** and related compounds on haspin kinase activity. Compounds were tested at 100 nM for 3 h. Error bars represent the SEM of duplicates.

Figure 3.

Dose-response curves showing the activity of **HSD972**, **HSD929** and compound **17** against haspin kinase. Experiment was done as a service by Reaction Biology Corp. Data was fitted to a non-linear regression equation using GraphPad Prism 5.0 software. Each data point represents the mean of duplicates.

Figure 4.
Inhibition of cell viability by **HSD972** and analogs. Cells were incubated with 10 μ M compounds and viability accessed after 72 h.

Figure 5.
Dose-response curves showing the inhibition of proliferation of **HSD929** and **HSD972** on **A)** HCT116 and **B)** HeLa cell lines. Every data point represent the mean of triplicates and the error bars represent the SEM.

Figure 6. Modeling of the active site interaction between haspin (PDB 3DLZ)[22] and **HSD972**. **A)** Hydrogen bond interaction between **HSD972** and hinge residue Glu606. **B)** Overlay of docked **HSD972** with crystalized ADP (magenta) in the ATP binding pocket of haspin. Both make hydrogen bonds to Glu606. An additional hinge region interaction between ADP and Gly608 is shown. **C)** Hydrophobic residues around the cyclohexyl ring B of **HSD972**. **D)** Hydrophobic patches above and below the cyclohexyl ring B in the active site. The atoms of the cyclohexyl ring are colored green. Docking was performed using GLIDE in the Schrodinger software package.

Figure 7.

The effect of **HSD972** on cell cycle progression in HCT116 cells after 24 h. An increase in cells in G2/M phase was observed with 13.5 μ M **HSD972** compared to DMSO [DMSO G2 (16.2%) versus HSD972 G2 (59.3%)].

Figure 8.
HSD972 causes an increase in caspase 3/7 activities in HCT116 cells. Data represent the normalized value to DMSO at each time point.

Scheme 1.

A one-flask synthesis of 3*H*-pyrazolo[4,3-*f*]quinoline-containing compounds, using HCl acid as a catalyst.