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Bis(hetero)aryl derivatives as unique kinesin spindle protein inhibitors

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ABSTRACT

Synthesis of 4-(4-*tert*-butylphenyl)pyridine analogues as kinesin spindle protein (KSP) inhibitors, SAR, cytotoxicity and mitotic arrest in HeLa cells are described. Interestingly, PVZB1194 showed potent KSP inhibition only in the presence of microtubules and distinct KSP localization from a known KSP inhibitor *S*-trytylcysteine analogue in mitosis. The observations would have resulted from a different molecular mechanism of KSP inhibition and suggest a novel biological regulation for KSP in mitosis.

The kinesin spindle protein (KSP), also known as homo sapiens Eg5, is a member of the kinesin-5 family in motor proteins.[1] In mitotic cells, KSP localizes at spindle poles and along interpolar spindle microtubules.[2] The spindle-bound KSP plays an essential role during the early stage of mitosis, separating the duplicated centrosome and thus encouraging assembly of the bipolar mitotic spindle array.[3] Failure of centrosome separation through KSP dysfunction results in the activation of the mitotic checkpoint and prolonged mitotic arrest, followed by apoptosis.[4] As KSP is absent in postmitotic neurons and is likely to act only in dividing cells, its inhibitors might provide better clinical therapy in the treatment of human malignancies than microtubule inhibitors such as paclitaxel or the vinca alkaloids.[5]

Since the discovery of monastrol,[6] a large number of KSP inhibitors (e.g., compound A)[7] and a KSP modulator KSPA-1[8] have been reported, some of which have shown preclinical proof of concept as anticancer drugs (Chart 1)[9]; many bind to the same allosteric loop 5 (L5) region of the motor domain in KSP as monastrol. The following five KSP inhibitors are currently undergoing clinical studies: ispinesib mesylate,[10] SB-743921,[11] MK-0731,[12] ARRY-520,[13] and EMD-534085.[14]

We have found that 4-(4-*tert*-butylphenyl)pyridine (**1**) exhibits KSP ATPase inhibition (IC_{50} = 1.00 μmol/L) and cytotoxicity to HeLa cells (IC_{50} = 88 μmol/L) by our screening campaign. Further structural modification was conducted and the detailed biological evaluations revealed significant insights into multiple and strict KSP regulations. Related compounds were disclosed recently[15] and this prompts us to disclose herein our own results with unique biological effects.

We were inspired that the nitrogen atom might have an essential role for KSP ATPase inhibition from the simple structure of compound **1**. Based on our hypothesis, bis(hetero)aryl analogues with various substituents characterizing hydrogen donors or acceptors were synthesized. Alongside these modifications, replacement of the 4-*tert*-butyl group with a related substituent was also investigated. The synthetic procedure is summarized in

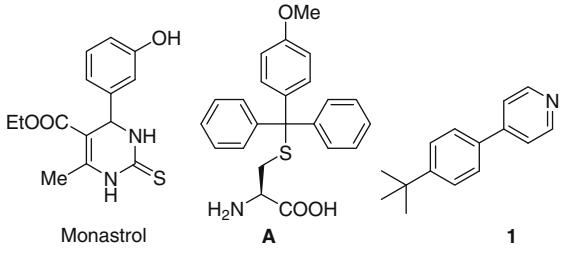
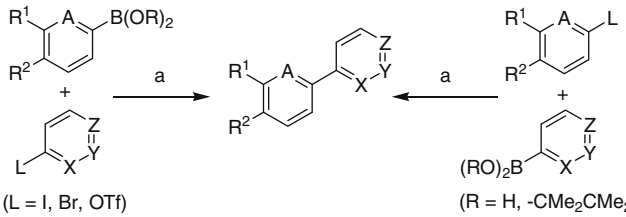


Chart 1. Structure of known KSP inhibitors and compound **1**.



Scheme 1. Reagents and conditions: (a) $PdCl_2(dppf)CH_2Cl_2$, K_3PO_4, 1,4-dioxane, DMF, heat.

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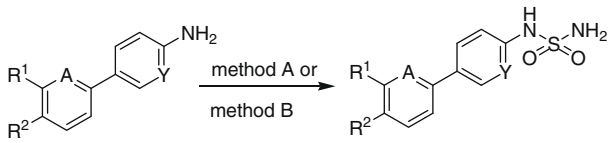


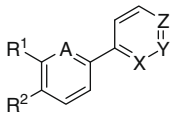
Scheme 2. Reagents and conditions: method A: (i) $ClSO_2NCO$, *t*-BuOH, Et_3N, CH_2Cl_2, 0 °C; (ii) substrate; (iii) CF_3COOH, CH_2Cl_2; method B: sulfamide (excess), 1,4-dioxane, reflux.

Schemes 1 and 2. Fundamentally, Suzuki–Miyaura coupling for the corresponding arylhalide/triflate and arylboronic acid/ester provided target molecules. Compounds **22**–**26** were synthesized by sulfamoylation of the corresponding amines with *tert*-butyl chlorosulfonylcarbamate, followed by TFA treatment (method A), or excess sulfamide directly (method B) (Scheme 2). The practical synthetic methods were also used to obtain some analogues.

The inhibitory activity on KSP ATPase[7] and cytotoxicity to HeLa cells[7] are summarized in Table 1. With regard to compounds responsible for exchanging the position of the *tert*-butyl group or nitrogen atom on pyridine (compounds **1**–**5**), 2-*tert*-butyl (**3**) or 2-pyridyl (**5**) was completely devoid of activity. These results indicate that the orientation of the *tert*-butyl group and N atom and/or the conformation of both (hetero)aryl rings are important for interaction with KSP. Among them, the combination of 3-*tert*-butyl and 4-pyridyl (e.g., compound **2**) demonstrated slightly improved activity. Movement of the nitrogen atom outside of the ring, namely, 4-cyanophenyl analogue (**6**) retained activity. Here, we exchanged the *tert*-butyl group for the related groups. Analogue of 4-CF_3 (**7**) reduced activity; however, incorporation of a 3-F atom (compound **8**) recovered KSP ATPase inhibitory activity equipotent to parent compound **6**. The cytotoxicity of compound **8** (IC_{50} = 13 μmol/L) was more potent than that of compound **6** (IC_{50} = 63 μmol/L); therefore, we selected 3-F-4-CF_3 as substituents on the left phenyl ring for further exploration.

Next, various substituents with a feature of a hydrogen donor or acceptor were introduced at the 4-position on the right phenyl ring (**9**–**16**). Analogues with 4-OH (**9**), 4-NH_2 (**10**), and 4-CHO (**13**) were equipotent, while 4-CH_2OH (**11**) and 4-$COCH_3$ (**14**) were weaker than compound **6**. In addition, 4-CH_2NH_2 (**12**) and 4-$COOCH_3$ (**15**) were completely inactive. On the other hand, incorporation of 4-SO_2NH_2 (**16**; PVZB1194) enhanced KSP ATPase inhibition (IC_{50} = 0.12 μmol/L) and cytotoxicity (IC_{50} = 5.5 μmol/L). Thus, we focused on the synthesis of the related analogues (**17**–**24**) of PVZB1194. Sequential methylation (**17** and **18**) on the sulfonamide moiety gradually decreased activity. Analogues of 4-SO_2CH_3 (**20**), (DL)-4-$SOCH_3$ (**19**), and 4-$NHSO_2CH_3$ (**22**) showed reduced activity (but **20** and **22** were almost equipotent to parent compounds **1** and **6**), and extension to 4-$SO_2CH_2CH_3$ (**21**) abolished activity. Furthermore, compounds with sulfamide (**23**) showed potent KSP ATPase inhibition (IC_{50} = 0.02 μmol/L), and insertion of methylene (compound **24**) reduced activity. Disfavorably, compound **23** showed >30-fold discrepancy for the cytotoxicity. We thought that the very high lipophilicity should be reduced to improve solubility, cell permeability and protein binding issues. Based on this assumption, we prepared compounds **25** and **26**, whose pyridine rings were replaced from phenyl rings. Compound **25** showed potent KSP ATPase inhibition (IC_{50} = 0.04 μmol/L); however, the cytotoxicity was dramatically decreased (IC_{50} = 30 μmol/L). The expanded discrepancy might be due to the physicochemical property of pyr-

Table 1
Inhibitory activity on KSP ATPase and HeLa cell proliferation



Compound	A	R^1	R^2	X	Y	Z	IC_{50}[a] (μmol/L)	
							KSP ATPase	Cytotoxicity
1	CH	H	*t*-Bu	CH	CH	N	1.00	88
2	CH	*t*-Bu	H	CH	CH	N	0.42	32
3	C-*t*-Bu	H	H	CH	CH	N	>20	NT[b]
4	CH	H	*t*-Bu	CH	N	CH	6.80	>100
5	CH	H	*t*-Bu	N	CH	CH	>20	NT[b]
6	CH	H	*t*-Bu	CH	CH	C-CN	1.47	63
7	CH	H	CF_3	CH	CH	C-CN	5.49	NT[b]
8	CH	F	CF_3	CH	CH	C-CN	1.40	13
9	CH	F	CF_3	CH	CH	C-OH	2.14	<100
10	CH	F	CF_3	CH	CH	C-NH_2	2.47	NT[b]
11	CH	F	CF_3	CH	CH	C-CH_2OH	3.63	33
12	CH	F	CF_3	CH	CH	C-CH_2NH_2	>20	NT[b]
13	CH	F	CF_3	CH	CH	C-CHO	1.47	68
14	CH	F	CF_3	CH	CH	C-$COCH_3$	7.14	NT[b]
15	CH	F	CF_3	CH	CH	C-$COOCH_3$	>20	NT[b]
16 (PVZB1194)	CH	F	CF_3	CH	CH	C-SO_2NH_2	0.12	5.5
17	CH	F	CF_3	CH	CH	C-SO_2NHCH_3	4.61	42
18	CH	F	CF_3	CH	CH	C-$SO_2N(CH_3)_2$	>20	NT[b]
19	CH	F	CF_3	CH	CH	C-(DL)-$SOCH_3$	3.70	91
20	CH	F	CF_3	CH	CH	C-SO_2CH_3	0.86	23
21	CH	F	CF_3	CH	CH	C-$SO_2CH_2CH_3$	>20	NT[b]
22	CH	F	CF_3	CH	CH	C-$NHSO_2CH_3$	2.78	9.9
23	CH	F	CF_3	CH	CH	C-$NHSO_2NH_2$	0.02	0.69
24	CH	F	CF_3	CH	CH	C-$CH_2NHSO_2NH_2$	3.06	63
25	CH	F	CF_3	CH	N	C-$NHSO_2NH_2$	0.04	30
26 (PVZB1084)	N	H	CF_3	CH	CH	C-$NHSO_2NH_2$	0.70	1.9

[a] IC_{50} values were derived from dose–response curves generated from triplicate data points.
[b] Not tested.

idylsulfamide. In contrast, compound **26** (PVZB1084) demonstrated well-balanced properties, namely, equipotent activity both for KSP ATPase inhibition (IC_{50} = 0.70 μmol/L) and cytotoxicity (IC_{50} = 1.89 μmol/L), as we expected.

The observed SAR indicates that the hydrogen bond at the 4-position of the right (hetero)aryl moiety plays a critical role in KSP inhibition. We speculate that a hydrogen bond acceptor, such as CN and a pyridine-N atom, interacts via a water molecule, while direct interaction occurs for a hydrogen bond donor, such as $NHSO_2NH_2$ and SO_2NH_2.

To understand KSP inhibition and the consequent cytotoxicity induced by synthesized analogues in cellular molecular biology, we evaluated the mechanism of action of KSP inhibition, mitotic arrest and the molecular dynamics of essential players in mitosis. Figure 1 shows KSP ATPase inhibition with/without microtubules by PVZB1194 and compound A, which is believed to bind to the L5 region in KSP.[16] PVZB1194 demonstrated weaker KSP ATPase inhibition in the absence of microtubules, unlike compound A. In addition, the inhibiting property of PVZB1194 is the same as that of a new ATP competitive KSP inhibitor, GSK-1.[17] PVZB1194 did not bind to microtubules directly (data not shown). A group from GlaxoSmithKline and Cytokinetics reported in an in silico docking study that GSK-1 might bind to a novel allosteric site that is distinct from the allosteric L5 region.[16] Therefore, we believe that the binding domain of PVZB1194 is different from that of known L5 allosteric KSP inhibitors. We are now conducting X-ray crystallographic analysis of the KSP/PVZB1194 complex to confirm the binding site, and results will be published elsewhere in the near future.

Next, we evaluated the effects of several cytotoxic KSP inhibitors on mitosis. All compounds arrested HeLa cells in the M-phase and the 50% mitotic index induction (MI_{50}s)[7] concentration was similar to the IC_{50} of cytotoxicity (Table 2). Since these compounds did not inhibit ATPase activity of another four structurally and functionally related mitotic kinesins, namely, CENP-E, kid, MKLP1 and KIF4 (data not shown),[7] cytotoxicity would be due to specific inhibition of KSP.

Finally, we investigated the dynamics of chromosomes, microtubules and KSP in HeLa cells by treatment with PVZB1194 or compound A.[18] As we expected, PVZB1194 induced the 'monoastral' phenotype, which is typically observed under KSP suppression (Fig. 2). Surprisingly, KSP distribution in mitotic arrested HeLa cells was quite different between PVZB1194 and compound A, despite the same localization of chromosomes and microtubules for both KSP inhibitors (Fig. 3). In the early mitotic phase, centrosomal KSP localization is normally observed and treatment with both KSP inhibitors disturbed it (this indicates endogenous KSP inhibition); PVZB1194 restricted KSP localization into the central portion of the cells, whereas compound A forced diffused KSP localization over the cells at almost the same concentration of MI_{50}. PVZB1194 is a specific KSP inhibitor (vide supra) and it inhibits KSP in a microtubule-dependent manner (Fig. 1). KSP is recruited around centrosomes and moves to plus end of microtubules in mitosis[3]; therefore, the restricted localization might suggest that PVZB1194 inhibits KSP moving immediately after KSP recruitment. As another possibility, KSP might be regulated by an unknown molecular

Table 2
Mitotic arrest in HeLa cells induced by selected analogues

Compound	MI^a_{50} (μmol/L)
8	9.9
PVZB1194	9.4
25	30
PVZB1084	11

[a] MI_{50} values were derived from dose–response curves generated from triplicate data points.

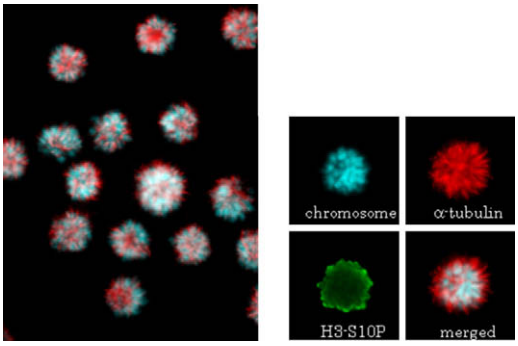


Figure 2. HeLa cells with monoasters after treatment with PVZB1194 (left: 36 μmol/L, right: 18 μmol/L). Chromosomes are colored in blue, α-tubulin in red and Ser10 phosphorylated histone H3 in green.

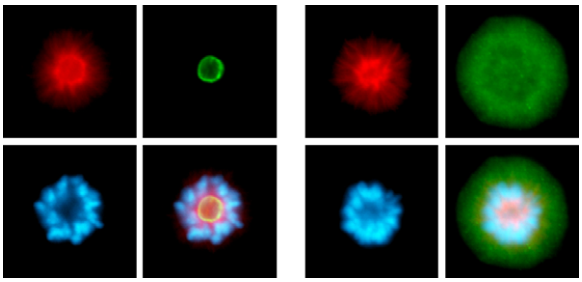


Figure 3. KSP localization in HeLa cells by treatment with PVZB1194 (16 μmol/L; left) and compound A (0.5 μmol/L; right). α-Tubulin is colored in red, chromosome in blue and KSP in green.

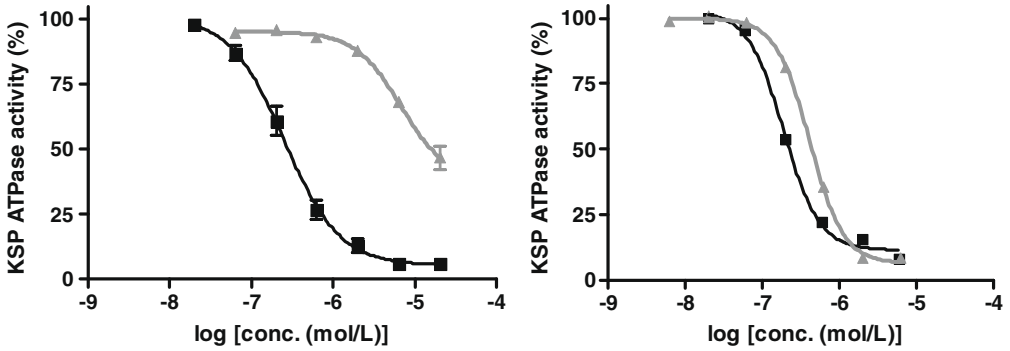


Figure 1. KSP ATPase inhibition with (black square) or without (gray triangle) microtubule by PVZB1194 (left) and compound A (right).

mechanism in mitosis, and KSP inhibitors with different molecular mechanisms could possibly distinguish it. Elucidation of the mode of action of PVZB1194 is now ongoing.

In conclusion, structural modification of KSP inhibitor 4-(4-*tert*-butylphenyl)pyridine (**1**) provided PVZB1194, and well-balanced PVZB1084. PVZB1194 potently inhibited KSP in a microruble-dependent manner and induced a monoastral phenotype to arrest mitotic progression as expected, while surprisingly, abnormal KSP localization was distinct from that induced by the known KSP inhibitor A. We suggest that KSP inhibitors with different mechanisms of action represented herein contribute to further elucidation of the cellular function of KSP in mitosis.

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18. HeLa cells treated with KSP inhibitors were fixed by the conventional paraformaldehyde/methanol method, and stained immunologically. DNA/chromatin, microtubules, mitotic chromatin and KSP were stained by DAPI, monoclonal anti-α-tubulin (DM1A) antibody (Sigma), anti-phospho-Histon H3 (Ser10) antibody (Upstate) and anti-KSP antibody (Cytoskeleton), respectively. Fluorescent images were observed under an Olympus IX71 microscope with a DP30BW CCD camera.