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Optimization of Diaryl Amine Derivatives as Kinesin Spindle Protein Inhibitors

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Abstract

Structure–activity relationship studies of diaryl amine-type KSP inhibitors were carried out. Diaryl amine derivatives with a pyridine ring or urea group were less active when compared with the parent carboline and carbazole derivatives. Optimization studies of a lactam-fused diphenylamine-type KSP inhibitor revealed that the aniline NH group and 3-CF₃ phenyl group were indispensable for potent KSP inhibition. Modification with a seven-membered lactam-fused phenyl group and a 4-(trifluoromethyl)pyridin-2-yl group improved aqueous solubility while maintaining potent KSP inhibitory activity. From these studies, we identified novel diaryl amine-type KSP inhibitors with a favorable balance of potency and solubility.

Keywords: diaryl amine, kinesin spindle protein, aqueous solubility

1. Introduction

Kinesins constitute a superfamily of molecular motor proteins to move along microtubules.¹ Mitotic kinesins are involved in cell division.² Non-mitotic kinesins are principally involved in intracellular transport of organelles and vesicles.³ The kinesin spindle protein (KSP; also known as Eg5) is the mitotic kinesin that belongs to the kinesin-5 family. The structure of KSP is comprised of three parts: an N-terminal motor domain, a central α-helical coiled coil stalk domain, and a C-terminal tail domain.⁴ The N-terminal motor domain contains a catalytic site for ATP hydrolysis and microtubule binding region. KSP moves toward the plus end of the microtubule, just like other kinesins with an N-terminal motor domain, using the energy generated from the hydrolysis of ATP.⁵ The KSP movement is required for centrosome separation and bipolar spindle formation during cell division. Inhibition of KSP leads to mitotic arrest in the prometaphase with the formation of the monopolar spindle and subsequent apoptotic cell death.⁶⁻⁹ Therefore, KSP inhibitors are
expected to be favorable agents for cancer chemotherapy without neurotoxic side effects.\textsuperscript{10-13}

Recently, we reported that carbazole derivative 1 with the 2-CF\textsubscript{3} group showed potent KSP inhibitory activity (Figure 1).\textsuperscript{14} Carbazole derivatives, with a lactam ring (2) or urea group (4c), and the \(\beta\)-carboline derivative 3a were also identified as highly potent KSP inhibitors by structure–activity relationship studies of 1.\textsuperscript{15} However, these inhibitors exhibited limited solubility in the aqueous solvents employed for in vivo studies. To satisfy the potent inhibitory activity requirements as well as better solubility in aqueous solution, we have designed diphenylamine derivatives such as 5a by modification of the planar carbazole-type inhibitor 2.\textsuperscript{16} Diphenylamine 5a exhibited better solubility than carbazole 2 while maintaining potent KSP inhibitory activity. Structural analysis by single crystal X-ray diffraction studies and free energy calculations demonstrated that the improved solubility of 5a is attributed to fewer van der Waals interactions in the crystal packing, as well as a hydrogen-bond acceptor nitrogen in the aniline moiety for favorable solvation. Interestingly, compound 5a possibly binds to the interface of the \(\alpha\)4 and \(\alpha\)6 of KSP in an ATP-competitive manner, whereas most KSP inhibitors (e.g., monastrol, S-trityl-L-cysteine) bind to the allosteric pocket formed by helices \(\alpha\)2, \(\alpha\)3 and loop L5 to show ATP-uncompetitive behavior.\textsuperscript{17-19} Replacing the right-hand 3-CF\textsubscript{3}-phenyl group in 5a with a pyridine ring provided a more soluble KSP inhibitor 6; however, this compound showed slightly lower potency than 5a.\textsuperscript{16} In this article, we describe the structure–activity relationship study for novel diaryl amine-type KSP inhibitors with high potency and aqueous solubility. For this purpose, we performed: (i) modification of ring-fused indoles such as 3a and 4c using the same approach employed for the development of 5a and (ii) intensive optimization studies of diphenylamine 5a.

2. Results and discussion

2.1. Investigation of diaryl amine-type KSP inhibitors by modification of ring-fused
scaffolds

We speculated that the poor solubility of carboline and carbazole derivatives would be attributable to the significant intermolecular interactions in the crystals (e.g., π–π stacking interactions) as seen for compound 2.16 To disrupt the possible crystal packing of compounds 3a and 4c, the design of less planar analogs was expected to be a promising approach.20 Therefore, we designed diaryl amine derivatives 7 and 8, in which the pyrrole C–C bond in the central part of carbolines 3 and carbazoles 4 was cleaved (Figure 2). Diaryl amines 7a,b with a pyridine ring were designed based on carbolines 3a,b with potent KSP inhibitory activity (Figure 2A). Diphenylamines 8a–f with a nitro, amino or urea group at the 3- or 4-position on the left-hand phenyl ring were similarly investigated, which represent the cleaved analogs of carbazoles 4b–f (Figure 2B). Diaryl amine derivatives 7a,b and 8a,d were prepared by palladium-catalyzed N-arylation using aryl bromides 9 and substituted anilines 10 (Scheme 1).21 For the preparation of compounds 8c,f with a urea group, nitro derivatives 8a,d were reduced to the corresponding amines 8b,e using Pd/C and ammonium formate, which were converted to the expected compounds 8c,f by KOCN.

First, KSP inhibitory activity of compounds 7a,b, with a pyridine ring in the left-hand part, was comparatively assessed with the parent carboline-type inhibitors 3a,b (Table 1). Unfortunately, the cleavage of the pyrrole C–C bond in carboline led to loss of KSP ATPase inhibitory activity at 6.3 μM. The solubility of these compounds was evaluated by a thermodynamic method.22 A mixture of EtOH–phosphate buffer (pH 7.4) (1:1) [50% EtOH] and phosphate buffer (pH 7.4) were employed as aqueous media.22 In these solutions, the parent carbazole-type inhibitor 1 was moderately soluble (0.424 mg/mL) and insoluble (<1 μg/mL), respectively. N-(Pyridin-3-yl)amine 7a showed the anticipated improvement in thermodynamic solubility, being 30 times more soluble in 50% EtOH (14.3 mg/mL) compared with the corresponding carboline 3a. N-(Pyridin-4-yl)amine 7b also exhibited
approximately 14 times greater solubility in 50% EtOH (24.0 mg/mL) than the parent carboline 3b. Of note, compound 7b had moderate solubility (264 μg/mL) even in phosphate buffer, which was 80 times or more soluble compared with 3b and 3a, respectively. Although these pyridinylamine derivatives 7a,b were inert in KSP inhibition, it was suggested that cleavage of the pyrrole C–C bond in carboline and carbazole derivatives could be a promising approach to improve the solubility in aqueous solution.

Diphenylamine derivatives 8a–f with a nitro, amino or urea group at the 3- or 4-position on the left-hand phenyl group were next examined (Table 2). The 3-substituted anilines 8a–c showed no KSP inhibitory activity at 6.3 μM. Among the 4-substituted analogs, compound 8f with a urea group exhibited moderate inhibitory activity (IC50 = 0.39 μM), while compounds 8d,e with a nitro or amino group had weak or no potency. The potency of 8f was approximately three times lower than that of the parent carbazole 4f.

2.2. Optimization studies of lactam-fused diaryl amine-type KSP inhibitors

Next, we investigated the structure–activity relationship and the further refinement of 5a for novel potent KSP inhibitors in terms of: (i) the linkage between the two aryl groups, (ii) the substituent on the right-hand phenyl group, (iii) the left-hand heterocycle, and (iv) the right-hand aromatic heterocycle (Figure 3).

A series of diaryl amine derivatives 5 and 11–14 were prepared by Buchwald–Hartwig N-arylation using aryl bromides or triflates 9 and substituted anilines 10, except for the compounds (11j,l,n and 12c,k) indicated below (Scheme 1).21 Diphenylether derivative 17a was obtained by treatment of phenol derivative 15 with diaryliodonium tetrafluoroborate 16 in the presence of KOt-Bu.23 Diphenylsulfide derivatives 20a,b were prepared by the copper-catalyzed C–S bond-forming reaction using aryl thiol 18 and CF3-substituted iodobenzenes 19a,b.24 Compounds 11j, 11l, 11n and 12k were obtained by simple
transformations including BBr₃-mediated demethylation of 11h, Zn-mediated reduction of 11k, saponification of 11m, and thiacarbonylation of 5a using Lawesson’s reagent, respectively. Compound 12c was prepared by treatment of 12a with O₂ in the presence of Pd(OAc)₂.

We initially investigated the type of heteroatom in the central part of diphenylamine 5a and the position of a substituent on the right-hand phenyl group (Table 3). Replacing the bridging NH group in compound 5a with oxygen (17a,b: ether) or sulfur (20a,b: thioether) resulted in a significant reduction or loss of KSP inhibitory activity. This indicates that the aniline NH group of 5a is an indispensable functional group as a hydrogen-bond donor, which is supported by our previous modeling study. Regarding the position of CF₃ group on the right-hand phenyl ring of 5a, 4-CF₃ compound 5b was approximately 7 times less potent than 5a and no KSP inhibition of 2-CF₃ compound 5c was observed.

Next, the structure–activity relationship was examined by replacing 3-substituents on the right-hand phenyl group in 5a (Table 4). Among compounds 11a–d with or without a 3-alkyl substituent, the tert-butyl derivative 11d exhibited the most potent inhibitory activity (IC₅₀ = 0.16 µM). The structure–activity relationship of the simple alkyl group correlated with that of the carbazole-type KSP inhibitors, suggesting that carbazoles (1 and 2) and diphenylamines (5a and 11d) may occupy the same binding site of KSP. Substitution with 3,5-di-CF₃ (11e), 3-phenyl (11f), 3-phenoxy (11g) and 3-methoxy (11h) groups were not effective. Introduction of a polar substituent such as 3-hydroxy (11j), 3-amino (11l), 3-methoxycarbonyl (11m), or 3-carboxylate (11n) also gave rise to inactive compounds. 3-Trifluoromethoxy (11i) and 3-nitro (11k) derivatives showed moderate inhibitory activity. It is inferred from these data that the substituent at the 3-position on the right-hand phenyl part is buried in a relatively large hydrophobic pocket of KSP. The possible binding mode of 11d to the interface of the α4 and α6 helices of KSP is shown in Figure 5. The tert-butyl group of 11d was buried in the
deep hydrophobic pocket formed by Tyr104, Gly296, Ile299, Thr300, Ile332, Tyr352, Ala353, and Ala356. Low desolvation energy of tert-butyl group compared with smaller alkyl groups may also contribute to the favorable binding of 11d to KSP.

In the left part of the molecule, the position of accessory amide group was crucial for the potency (Table 5). Compounds 12a–d with an amide group at the 3-position were less active or inactive, while the parent compound 5a with the 4-position amide group showed potent KSP inhibitory activity. A five-membered ring thiocarbamate 12g exhibited moderate inhibitory activity (IC₅₀ = 0.81 µM) in contrast to the ineffectiveness in lactam derivative 12e and carbamate derivative 12f, suggesting that the introduction of a sulfur atom into the lactam ring had favorable effects on the bioactivity. The addition of a sulfur atom (12i) into the six-membered lactam of 5a also maintained potent KSP inhibitory activity (IC₅₀ = 0.051 µM), whereas an oxygen atom (12h) decreased the inhibitory activity. The loss of activity in the N-methylamide derivative 12j indicates that the lactam NH group at this position is essential for KSP inhibition. Compound 12k, with a thioamide group, had slightly reduced potency (IC₅₀ = 0.19 µM) compared with 5a. Compound 12l, with a seven-membered lactam, showed approximately equipotent KSP inhibitory activity (IC₅₀ = 0.050 µM) to 5a, suggesting that some flexibility of the lactam carbonyl placement is tolerated. Substitution with a fluorine group on the 5- or 6-position (12m,n) resulted in a reduction in the inhibitory activity, suggesting that modification at these positions was inappropriate for favorable interactions with KSP.

2.3. Analysis of aqueous solubility of potent KSP inhibitors and further optimization

The thermodynamic solubility of potent diphenylamine derivatives 12i,l in aqueous media was next evaluated (Table 6).²² Diphenylamine 12i with a thiomorpholin-3-one structure was slightly less soluble (1.70 mg/mL in 50% EtOH) than the parent compound 5a. The longer
retention time on a reversed-phase HPLC column (28.2 min) of compound 12i indicated that the introduction of a sulfur atom into the lactam ring of 5a resulted in the increased hydrophobicity, thereby lowering the solubility in aqueous media. Diphenylamine 12l with a seven-membered lactam ring was approximately four times more soluble (7.39 mg/mL in 50% EtOH) than 5a, although a high CLogP value and HPLC retention time indicated higher hydrophobicity. The lower melting point of compound 12l (140 °C) suggested that the weak crystal packing mainly contributed to the observed improvement in solubility. These results indicated that the seven-membered lactam ring on the left-hand phenyl group was a promising structural unit for the development of KSP inhibitors that have a favorable balance of bioactivity and aqueous solubility.

As such, we identified benzothiomorpholin-3-one 12i and benzoazepin-2-one 12l as potent KSP inhibitors using structure–activity relationship studies of fused heterocycles on the left-hand phenyl group. Compound 12l represents the lead compound for further structural refinements because of the improved solubility. Separately, examination of the right-hand phenyl group identified 6-pyridine derivative 6 with potent KSP inhibitory activity and improved aqueous solubility. On the basis of these promising components, two diaryl amine derivatives were then designed (Figure 4). Diaryl amine 13 was designed based on compound 12i with a thiomorpholin-3-one structure and compound 6 with a 6-pyridine ring. Diaryl amine 14 was similarly designed based on compounds 12l and 6.

Thiomorpholin-3-one 13 exhibited the most potent KSP inhibitory activity (IC50 = 0.035 μM) among the diaryl amine-type inhibitors that were examined in this study (Table 6). However, compound 13 was less soluble in 50% EtOH (0.669 mg/mL) and in phosphate buffer (1.11 μg/mL) than the parent compound 6. The high melting point of compound 13 (217 °C) indicated that the tight crystal packing might cause this decrease in aqueous solubility. Benzoazepin-2-one 14 maintained highly potent KSP inhibitory activity (IC50 =
0.050 μM) as seen for the parent compounds 5a, 6 and 12l. Compound 14 exhibited greater solubility in 50% EtOH (4.82 mg/mL) and in phosphate buffer (8.07 μg/mL) than the corresponding compound 6. Of note, solubility of compound 14 in phosphate buffer was remarkably improved, as we expected, in comparison with compound 12l (less than 1 μg/mL), although 14 was less soluble than 12l in 50% EtOH. Taken together, compound 14 was identified to be a novel KSP inhibitor with a favorable balance of potency and aqueous solubility.

Compounds 5a, 6, 12l, 13 and 14 were tested for their inhibitory effect on the proliferation of cancer cell lines: lung cancer cells A549, colorectal cancer cells HCT-116, and breast cancer cells MCF-7 (Table 7). Cells were treated with increasing concentrations of the compounds, and viabilities were measured by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. All the tested diaryl amine derivatives were shown to be effective against these cell lines. In particular, thiomorpholin-3-one derivative 13 with the highest KSP inhibitory activity was found to be the most potent against all the cell lines tested.26

3. Conclusions

We have performed structure–activity relationship studies for the development of novel KSP inhibitors using carbolines 3, carbazoles 4 and diphenylamine 5a as the lead compounds. Unfortunately, the nonplanar analogs 7 and 8 of planar carbolines 3 and carbazoles 4 decreased the potency for KSP inhibition. Optimization studies of diphenylamine 5a revealed that bridging NH group, 3-CF3 group on the right-hand phenyl group, and the lactam amide group at the 4-position on the left-hand phenyl group contributed to the potent KSP inhibitory activity. Further investigations provided novel KSP inhibitors 13 with the most potent inhibitory activity and 14 with the optimal balance of potency and aqueous solubility in this
4. Experimental

4.1. Synthesis

4.1.1. General methods

$^1$H NMR spectra were recorded using a JEOL AL-400 or a JEOL ECA-500 spectrometer. Chemical shifts are reported in $\delta$ (ppm) relative to Me$_4$Si as an internal standard. $^{13}$C NMR spectra were referenced to the residual DMSO signal. Exact mass (HRMS) spectra were recorded on a JMS-HX/HX 110A mass spectrometer. Melting points were measured by a hot stage melting point apparatus (uncorrected). For flash chromatography, Wakogel C-300E (Wako) or Chromatorex® NH was employed. For analytical HPLC, a Cosmosil 5C18-ARII column (4.6 x 250 mm, Nacalai Tesque, Inc., Kyoto, Japan) was employed with a linear gradient of CH$_3$CN containing 0.1% (v/v) TFA at a flow rate of 1 mL/min on a Shimadzu LC-10ADvp (Shimadzu Corp., Ltd., Kyoto, Japan), and eluting products were detected by UV at 254 nm. The purity of the compounds was determined by combustion analysis or HPLC analysis (> 95%).

4.1.2. General procedure of $N$-arylation for preparation of diaryl amine compounds: synthesis of 6-{[3-(trifluoromethoxy)phenyl]amino}-3,4-dihydroquinolin-2(1H)-one (11i)

Toluene (4.5 mL) was added to a flask containing 6-bromo-3,4-dihydroquinolin-2(1H)-one (300 mg, 1.33 mmol), 3-(trifluoromethoxy)aniline (231 $\mu$L, 1.73 mmol), Pd$_2$(dba)$_3$ (15.2 mg, 0.02 mmol), 2-dicyclohexylphosphino-2',4',6'-triisopropylbiphenyl (31.6 mg, 0.07 mmol) and NaOt-Bu (192 mg, 2.00 mmol) under an argon atmosphere. The mixture was stirred at 100 °C for 9 h. After cooling, the reaction mixture was diluted with EtOAc, and filtered through a pad of Celite. The filtrate was concentrated in vacuo. Crude material was purified by flash
chromatography with n-hexane/EtOAc (2:3) to afford the desired diaryl amine 11i (292 mg, 68% yield): pale yellow solid; mp 160-162 °C; IR (neat) cm⁻¹: 1667 (C=O), 3219 (NH), 3315 (NH); ¹H NMR (500 MHz, DMSO-d₆) δ 2.43 (t, J = 6.9 Hz, 2H; CH₂), 2.85 (t, J = 6.9 Hz, 2H; CH₂), 6.63 (d, J = 8.0 Hz, 1H; Ar), 6.79 (s, 1H; Ar), 6.81 (d, J = 8.0 Hz, 1H; Ar), 6.91-6.96 (m, 3H; Ar), 7.25 (t, J = 8.0 Hz, 1H; Ar), 8.25 (s, 1H; NH), 9.99 (s, 1H; NH); ¹³C NMR (125 MHz, DMSO-d₆) δ 25.0, 30.4, 106.3, 109.6, 113.1, 115.8, 118.7, 119.5, 120.1 (q), 124.7, 130.7, 133.1, 136.1, 146.9, 149.4, 169.8; Anal. calcd for C₁₆H₁₃F₃N₂O₂: C, 59.63; H, 4.07; N, 8.69. Found: C, 59.51; H, 4.12; N, 8.59.

4.1.3. N¹-[3-(Trifluoromethyl)phenyl]benzene-1,3-diamine (8b)

To a stirred solution of 3-nitro-N¹-[3-(trifluoromethyl)phenyl]aniline 8a (190 mg, 0.67 mmol) and ammonium formate (509 mg, 8.07 mmol) in EtOH (2.2 mL) at room temperature was added 10% Pd/C (35.8 mg, 0.03 mmol). The mixture was heated under reflux for 2 h. After cooling, the reaction mixture was diluted with EtOAc, and filtered through a pad of Celite. The filtrate was concentrated in vacuo. Crude material was purified by flash chromatography with n-hexane/EtOAc (3:1) to afford compound 8b (165 mg, 97%): brown oil; IR (neat) cm⁻¹: 3035 (NH), 3377 (NH); ¹H NMR (400 MHz, DMSO-d₆) δ 4.96 (s, 2H; NH₂), 6.19 (d, J = 8.0 Hz, 1H; Ar), 6.28 (d, J = 8.0 Hz, 1H; Ar), 6.37 (s, 1H; Ar), 6.92 (t, J = 8.0 Hz, 1H; Ar), 7.01 (d, J = 8.0 Hz, 1H; Ar), 7.22 (s, 1H; Ar), 7.25 (d, J = 8.2 Hz, 1H; Ar), 7.37 (t, J = 8.0 Hz, 1H; Ar), 8.13 (s, 1H; NH); ¹³C NMR (125 MHz, DMSO-d₆) δ 104.0, 106.5, 107.8, 111.3, 114.3, 118.8, 124.3 (q), 129.6, 129.8 (q), 130.0, 142.5, 145.2, 149.7; HRMS (FAB): m/z calcd for C₁₃H₁₁F₃N₂ (M⁺) 252.0874; found: 252.0874.

4.1.4. 1-(3-[3-(Trifluoromethyl)phenyl]amino)phenyl]urea (8c)

To a stirred solution of N¹-[3-(trifluoromethyl)phenyl]benzene-1,3-diamine 8b (80.0 mg,
0.32 mmol) in AcOH (4.0 mL) was added KOCN (77.2 mg, 0.95 mmol) and water (80.0 µL). The reaction mixture was stirred at room temperature for 18 h, then evaporated to dryness under vacuum. Crude material was purified by flash chromatography with amino silica gel with CHCl₃/MeOH (20:1 to 10:1) to afford compound 8c (31.9 mg, 34% yield): yellow oil; IR (neat) cm⁻¹: 1661 (C=O), 3225 (NH), 3351 (NH); ¹H NMR (500 MHz, DMSO-d₆) δ 5.82 (br, 2H; NH₂), 6.66 (d, J = 8.0 Hz, 1H; Ar), 6.84 (d, J = 8.0 Hz, 1H; Ar), 7.06 (d, J = 8.0 Hz, 1H; Ar), 7.12 (t, J = 8.0 Hz, 1H; Ar), 7.28 (s, 1H; Ar), 7.30 (d, J = 8.0 Hz, 1H; Ar), 7.41 (s, 1H; Ar), 7.42 (t, J = 8.0 Hz, 1H; Ar), 8.50 (br, 2H; NH); ¹³C NMR (125 MHz, DMSO-d₆) δ 107.4, 110.7, 110.9, 111.5, 114.8, 118.9, 124.2 (q), 129.3, 129.8 (q), 130.1, 141.5, 142.2, 144.6, 155.8; HRMS (FAB): m/z calcd for C₁₄H₁₂F₃N₃O (M⁺) 295.0932; found: 295.0926.

4.1.5. 6-[(3-Hydroxyphenyl)amino]-3,4-dihydroquinolin-2(1H)-one (11j)

A suspension of 6-[(3-methoxyphenyl)amino]-3,4-dihydroquinolin-2(1H)-one 11h (500 mg, 1.86 mmol) in dry CH₂Cl₂ (5 mL) was cooled to -78 °C and then BBr₃ (7.45 mL of 1 M solution in CH₂Cl₂, 7.45 mmol) was added. After the mixture was stirred for 30 min at -78 °C, the stirring was continued for additional 18 h at room temperature. The reaction was quenched by addition of water (12 mL), and CH₂Cl₂ was removed under reduced pressure. The aqueous solution was neutralized by addition of aqueous NaOH, and then extracted three times with EtOAc. The organic layer was washed with brine, and dried over Na₂SO₄. The organic solvent was removed under reduced pressure and the crude residue was purified by flash chromatography with n-hexane/EtOAc (1:3) to afford compound 11j (232 mg, 49% yield): brown needle crystal; mp 213-214 °C; IR (neat) cm⁻¹: 1651 (C=O), 3222 (NH), 3321 (NH); ¹H NMR (500 MHz, DMSO-d₆) δ 2.41 (t, J = 7.4 Hz, 2H; CH₂), 2.81 (t, J = 7.4 Hz, 2H; CH₂), 6.15 (d, J = 8.0 Hz, 1H; Ar), 6.38 (d, J = 8.0 Hz, 1H; Ar), 6.39 (s, 1H; Ar), 6.74 (d, J = 8.0 Hz, 1H; Ar), 6.85 (d, J = 8.0 Hz, 1H; Ar), 6.89 (s, 1H; Ar), 6.94 (t, J = 8.0 Hz, 1H; Ar), 7.77 (s,
1H; NH), 9.07 (s, 1H; OH), 9.89 (s, 1H; NH); 13C NMR (125 MHz, DMSO-$d_6$) δ 25.1, 30.5, 102.2, 106.0, 106.6, 115.6, 117.4, 118.2, 124.4, 129.7, 131.7, 137.7, 145.8, 158.1, 169.7; Anal. calcd for C$_{15}$H$_{14}$N$_2$O$_2$: C, 70.85; H, 5.55; N, 11.02. Found: C, 71.11; H, 5.59; N, 10.89.

4.1.6. 6-[(3-Aminophenyl)amino]-3,4-dihydroquinolin-2(1H)-one (11l)

To a stirred solution of 6-[(3-nitrophenyl)amino]-3,4-dihydroquinolin-2(1H)-one 11k (62.0 mg, 0.22 mmol) in AcOH (2.2 mL) at room temperature was added zinc powder (102 mg, 1.56 mmol) portionwise. After being stirred at room temperature for 1 h, the reaction mixture was filtered through a pad of Celite and concentrated under vacuum. The residue was diluted with EtOAc, and the whole was washed with saturated NaHCO$_3$, brine, and dried over Na$_2$SO$_4$. The organic solvent was removed under reduced pressure and the crude residue was purified by flash chromatography with n-hexane/EtOAc (1:8) to afford compound 11l (41.6 mg, 75% yield): pale yellow solid; mp 196-198 ºC; IR (neat) cm$^{-1}$: 1662 (C=O), 3221 (NH), 3344 (NH); 1H NMR (500 MHz, DMSO-$d_6$) δ 2.40 (t, $J = 7.4$ Hz, 2H; CH$_2$), 2.80 (t, $J = 7.4$ Hz, 2H; CH$_2$), 4.93 (br, 2H; NH$_2$), 6.00 (d, $J = 8.0$ Hz, 1H; Ar), 6.15 (d, $J = 8.0$ Hz, 1H; Ar), 6.24 (s, 1H; Ar), 6.72 (d, $J = 8.0$ Hz, 1H; Ar), 6.80 (d, $J = 8.0$ Hz, 1H; Ar), 6.83 (d, $J = 8.0$ Hz, 1H; Ar), 6.86 (s, 1H; Ar), 7.58 (s, 1H; NH), 9.87 (s, 1H; NH); 13C NMR (125 MHz, DMSO-$d_6$) δ 25.2, 30.5, 101.4, 104.5, 105.7, 115.5, 116.9, 117.7, 124.3, 129.3, 131.2, 138.3, 145.0, 149.2, 169.6; HRMS (FAB): calcd for C$_{15}$H$_{15}$N$_3$O (M$^+$) 253.1215; found: 253.1213.

4.1.7. 3-[(2-Oxo-1,2,3,4-tetrahydroquinolin-6-yl)amino]benzoic acid (11n)

To a solution of methyl 3-[(2-oxo-1,2,3,4-tetrahydroquinolin-6-yl)amino]benzoate 11m (400 mg, 1.35 mmol) in 5.2 mL of MeOH/H$_2$O (3:1 v/v) was added LiOH·H$_2$O (170 mg, 4.05 mmol) at 0 ºC, then the solution was warmed to 50 ºC. After 1 h, the reaction mixture was acidified to below pH 2 using 1 M HCl, then EtOAc and brine were added to the mixture. The
organic extracts were washed with brine and dried over Na₂SO₄. The organic solvent was
removed under reduced pressure to afford compound **11n** (366 mg, 96% yield): white solid;
mp 259-261 °C; IR (neat) cm⁻¹: 1656 (C=O), 1684 (C=O), 3203 (NH), 3326 (NH); ¹H NMR
(500 MHz, DMSO-<d>⁶) δ 2.43 (t, J = 7.4 Hz, 2H; CH₂), 2.85 (t, J = 7.4 Hz, 2H; CH₂), 6.81 (d,
J = 8.0 Hz, 1H; Ar), 6.93 (d, J = 8.0 Hz, 1H; Ar), 6.94 (s, 1H; Ar), 7.17 (d, J = 8.0 Hz, 1H; Ar),
7.27-7.31 (m, 2H; Ar), 7.53 (s, 1H; Ar), 8.12 (s, 1H; NH), 9.98 (s, 1H; NH), 12.78 (br, 1H;
CO₂H); ¹³C NMR (125 MHz, DMSO-<d>⁶) δ 25.1, 30.4, 115.5, 115.8, 118.1, 118.9, 119.0, 119.2,
124.6, 129.3, 131.7, 132.6, 136.9, 145.2, 167.6, 169.8; HRMS (FAB): m/z calcd for
C₁₆H₁₄N₂O₃ (M⁺) 282.1004; found: 282.1011.

4.1.8. 7-[(3-Trifluoromethyl)phenyl]amino]quinolin-2(1H)-one (12c)

AcOH (1.0 mL) was added to a flask containing
7-[(3-trifluoromethyl)phenyl]amino})-3,4-dihydroquinolin-2(1H)-one **12a** (80.0 mg, 0.26
mmol) and Pd(OAc)₂ (22.4 mg, 0.10 mmol) and an oxygen balloon was connected to the
reaction vessel. After stirring for 2 h at 115 °C, the reaction mixture was cooled to room
temperature and concentrated *in vacuo*. The residue was purified by flash chromatography
with *n*-hexane/EtOAc (1:3 to 1:5) to afford **12c** (34.1 mg, 43% yield): white solid; mp
177-179 °C; IR (neat) cm⁻¹: 1655 (C=O), 3452 (NH); ¹H NMR (500 MHz, DMSO-<d>⁶) δ 6.24
(d, J = 9.2 Hz, 1H; C=CH), 6.88 (dd, J = 8.0, 2.3 Hz, 1H; Ar), 7.06 (d, J = 2.3 Hz, 1H; Ar),
7.23 (d, J = 8.0 Hz, 1H; Ar), 7.41 (s, 1H; Ar), 7.45 (d, J = 8.0 Hz, 1H; Ar), 7.50–7.53 (m, 2H;
Ar), 7.75 (d, J = 9.2 Hz, 1H; C=CH), 8.99 (s, 1H; NH), 11.53 (s, 1H; NH); ¹³C NMR (125
MHz, DMSO-<d>⁶) δ 99.7, 112.3, 113.1, 113.5, 116.7, 117.7, 121.0, 124.1 (q), 129.0, 130.0 (q),
130.4, 139.8, 140.5, 142.9, 144.5, 162.2; HRMS (FAB): m/z calcd for C₁₆H₁₁F₃N₂O [M + H]⁺
305.0902; found: 305.0905.
4.1.9. 6-\{3-(Trifluoromethyl)phenyl\}amino\]-3,4-dihydroquinoline-2(1H)-thione (12k)

To a stirred solution of 6-\{3-(trifluoromethyl)phenyl\}amino\]-3,4-dihydroquinoline-2(1H)-one 5a (50.0 mg, 0.16 mmol) in toluene (1.0 mL) was added Lawesson’s reagent (33.0 mg, 0.08 mmol) under an argon atmosphere. After stirring for 30 min at 110 °C, the reaction mixture was cooled to room temperature and concentrated \textit{in vacuo}. The residue was purified by flash chromatography with \textit{n}-hexane/EtOAc (3:1) to afford 12k (51.4 mg, 100% yield): yellow solid; mp 195–196 °C; IR (neat) cm\(^{-1}\): 1499 (C=S), 3361 (NH); \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 2.77 (t, J = 8.0 Hz, 2H; CH\(_2\)), 2.90 (t, J = 8.0 Hz, 2H; CH\(_2\)), 6.97 (s, 1H; Ar), 6.98 (d, J = 8.0 Hz, 1H; Ar), 7.04 (d, J = 8.0 Hz, 1H; Ar), 7.06 (d, J = 8.0 Hz, 1H; Ar), 7.20 (s, 1H; Ar), 7.27 (d, J = 8.0 Hz, 1H; Ar), 7.41 (d, J = 8.0 Hz, 1H; Ar), 8.49 (s, 1H; NH), 12.15 (s, 1H; NH); \(^{13}\)C NMR (125 MHz, DMSO-\(d_6\)) \(\delta\) 24.0, 38.9, 111.4, 114.9, 117.0, 117.3, 118.3, 118.5, 124.3 (q), 126.6, 130.0 (q), 130.3, 131.3, 138.3, 144.9, 197.3; HRMS (FAB): \(m/z\) calcd for C\(_{16}\)H\(_{13}\)F\(_3\)N\(_2\)S (M\(^+\)) 322.0752; found: 322.0758.

4.1.10. 7-\{4-(Trifluoromethyl)pyridin-2-yl\}amino\]-2H-benzo[b][1,4]thiazin-3(4H)-one (13)

Following the general procedure for 11i, compound 13 (48.5 mg, 12% yield) was synthesized from 7-bromo-2H-benzo[b][1,4]thiazin-3(4H)-one and 2-amino-4-(trifluoromethyl)pyridine: white solid; mp 217–218 °C; IR (neat) cm\(^{-1}\): 1688 (C=O), 3194 (NH); \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 3.45 (s, 2H; CH\(_2\)), 6.92 (d, J = 8.6 Hz, 1H; Ar), 6.99 (d, J = 5.2 Hz, 1H; Ar), 7.03 (s, 1H; Ar), 7.33 (dd, J = 8.6, 1.7 Hz, 1H; Ar), 7.81 (d, J = 1.7 Hz, 1H; Ar), 8.37 (d, J = 5.2 Hz, 1H; Ar), 9.43 (s, 1H; NH), 10.44 (s, 1H; NH); \(^{13}\)C NMR (125 MHz, DMSO-\(d_6\)) \(\delta\) 29.1, 106.2, 108.6, 117.1, 117.5, 117.7, 119.3, 123.0 (q), 131.7, 135.9, 137.6 (q), 149.3, 156.1, 164.8; HRMS (FAB): \(m/z\) calcd for C\(_{14}\)H\(_{10}\)F\(_3\)N\(_3\)OS (M\(^+\)) 325.0497; found: 325.0497.
4.1.11. 7-[(4-(Trifluoromethyl)pyridin-2-yl)amino]-1,3,4,5-tetrahydro-2H-benzo[b]azepin-2-one (14)

Following the general procedure for 11i, compound 14 (16.7 mg, 18% yield) was synthesized from 7-bromo-1,3,4,5-tetrahydro-2H-benzo[b]azepin-2-one and 2-amino-4-(trifluoromethyl)pyridine: pale yellow solid; mp 185–187 °C; IR (neat) cm⁻¹: 1688 (C=O), 2936 (NH); ¹H NMR (500 MHz, DMSO-d₆) δ 2.09–2.16 (m, 4H; CH₂ × 2), 2.67 (t, J = 6.9 Hz, 2H; CH₂), 6.92 (d, J = 8.0 Hz, 1H; Ar), 6.98 (d, J = 5.2 Hz, 1H; Ar), 7.07 (s, 1H; Ar), 7.53 (d, J = 8.0 Hz, 1H; Ar), 7.55 (s, 1H; Ar), 8.37 (d, J = 5.2 Hz, 1H; Ar), 9.36 (s, 1H; NH), 9.42 (s, 1H; NH); ¹³C NMR (125 MHz, DMSO-d₆) δ 27.8, 30.1, 32.7, 106.0, 108.4, 117.4, 119.6, 121.9, 122.0, 123.0 (q), 132.7, 134.1, 137.4, 137.5 (q), 149.3, 156.3, 173.1; HRMS (FAB): m/z calcd for C₁₆H₁₄F₃N₃O (M⁺) 321.1089; found: 321.1092.

4.1.12. 6-[3-(Trifluoromethyl)phenoxy]-3,4-dihydroquinolin-2(1H)-one (17a)

To a suspension of KOT-Bu (60.5 mg, 0.54 mmol) in DMF (2.1 mL) was added 6-hydroxy-3,4-dihydroquinolin-2(1H)-one 15 (80.0 mg, 0.49 mmol) at 0 °C and the reaction mixture was stirred at this temperature for 15 min. Bis(3-trifluoromethylphenyl)iodonium tetrafluoroborate 16 (259 mg, 0.51 mmol) was added in one portion and the reaction mixture was stirred at 40 °C for 1 h, then quenched with H₂O at 0 °C and extracted into CHCl₃. The organic layer was washed with brine, and dried over Na₂SO₄. The organic solvent was removed under reduced pressure and the crude residue was purified by flash chromatography with n-hexane/EtOAc (1:1) to afford compound 17a (39.9 mg, 27% yield): white solid; mp 158-159 °C; IR (neat) cm⁻¹: 1681 (C=O), 3203 (NH); ¹H NMR (500 MHz, DMSO-d₆) δ 2.44 (t, J = 7.4 Hz, 2H; CH₂), 2.87 (t, J = 7.4 Hz, 2H; CH₂), 6.91 (s, 2H; Ar), 6.99 (s, 1H; Ar), 7.20–7.23 (m, 2H; Ar), 7.42 (d, J = 8.0 Hz, 1H; Ar), 7.58 (t, J = 8.0 Hz, 1H; Ar), 10.12 (s, 1H;
13C NMR (125 MHz, DMSO-d6) δ 24.7, 30.0, 113.6, 116.3, 118.7, 119.1, 119.5, 121.0, 123.7 (q), 125.7, 130.6 (q), 131.2, 135.3, 149.6, 158.4, 169.9; HRMS (FAB): m/z calcd for C16H12F3NO2 (M+) 307.0820; found: 307.0826.

4.1.13. 6-[[3-(Trifluoromethyl)phenyl]thio]-3,4-dihydroquinolin-2(1H)-one (20a)

2-Propanol (1.0 mL) was added to a flask containing 6-mercapto-3,4-dihydroquinolin-2(1H)-one 18 (50.0 mg, 0.28 mmol), 3-trifluoromethylbromobenzene 19a (40.4 µL, 0.28 mmol), CuI (32.0 mg, 0.17 mmol), ethylene glycol (31.2 µL, 0.56 mmol) and K2CO3 (96.7 mg, 0.70 mmol) under an argon atmosphere. The mixture was stirred at 80 °C for 13 h. After cooling, the reaction mixture was diluted with EtOAc, and filtered through a pad of Celite. The filtrate was concentrated in vacuo. Crude material was purified by flash chromatography with n-hexane/EtOAc (3:2) to afford compound 20a (6.2 mg, 7% yield): white solid; mp 102-104 °C; IR (neat) cm⁻¹: 1676 (C=O), 3387 (NH); 1H NMR (500 MHz, DMSO-d6) δ 2.53 (t, J = 7.4 Hz, 2H; CH2), 2.96 (t, J = 7.4 Hz, 2H; CH2), 7.00 (d, J = 8.0 Hz, 1H; Ar), 7.40 (d, J = 8.0 Hz, 1H; Ar), 7.42-7.45 (m, 2H; Ar), 7.49 (s, 1H; Ar), 7.58-7.59 (m, 2H; Ar), 10.34 (s, 1H; NH); 13C NMR (125 MHz, DMSO-d6) δ 24.4, 30.0, 116.4, 122.4, 122.6, 123.0, 123.7 (q), 125.4, 129.9 (q), 130.3, 131.0, 133.5, 133.7, 139.5, 140.1, 170.1; HRMS (FAB): m/z calcd for C16H12F3NOS (M⁺) 323.0592; found: 323.0591.

4.2. KSP ATPase assay

The microtubule-stimulated KSP ATPase reaction was performed in a reaction buffer [20 mM PIPES-KOH (pH 6.8), 25 mM KCl, 2 mM MgCl2, 1 mM EGTA-KOH (pH 8.0)] containing 38 nM of the bacteria-expressed KSP motor domain (1–369) fused to histidine-tag at the carboxyl-terminus and 350 nM microtubules in 96-well half-area plates (Corning). Each
chemical compound in DMSO at different concentrations was diluted 12.5-fold with the chemical dilution buffer [10 mM Tris-OAc (pH 7.4), 0.04% (v/v) NP-40]. After pre-incubation of 9.7 µL of the enzyme solution with 3.8 µL of each chemical solution at 25 °C for 30 min, the ATPase reaction was initiated by the addition of 1.5 µL of 0.3 mM ATP solution, and followed by incubation at 25 °C for further 15 min. The reaction was terminated by the addition of 15 µL of the Kinase-Glo Plus reagent (Promega). The ATP consumption in each reaction was measured as the luciferase-derived luminescence by ARVO Light (PerkinElmer). At least three experiments were performed per condition and the averages and standard deviations of inhibition rates in each condition were evaluated to determine IC50 values using the GraphPad Prism software.

### 4.3. Growth inhibition assay

A549, HCT-116 and MCF-7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Sigma), McCoy’s 5A medium (GIBCO) and Eagle's minimal essential medium (EMEM, Wako), respectively, supplemented with 10% (v/v) fetal bovine serum at 37 °C in a 5% CO2-incubator. Growth inhibition assays using these cells were performed in 96-well plates (BD Falcon). A549, HCT-116 and MCF-7 cells were seeded at 500, 5000 and 5000 cells/well in 50 µL of culture media, respectively, and were cultured for 6 h. Chemical compounds in DMSO were diluted 250-fold with the culture medium in advance. Following the addition of 40 µL of the fresh culture medium to the cell cultures, 30 µL of the chemical diluents were also added. The final volume of DMSO in the medium was equal to 0.1% (v/v). The cells under chemical treatment were incubated for further 72 h. The wells in the plates were washed twice with the cultured medium without phenol-red. After 1 h incubation with 100 µL of the medium, the cell culture in each well was supplemented with 20 µL of the MTS reagent (Promega), followed by incubation for additional 40 min. Absorbance at 490 nm of
each well was measured using a Wallac 1420 ARVO SX multilabel counter (Perkin Elmer). At least three experiments were performed per condition and the averages and standard deviations of inhibition rates in each condition were evaluated to determine IC₅₀ values using the GraphPad Prism software.

4.4. Thermodynamic solubility in aqueous solution

An equal volume of a mixture of 1/15 M phosphate buffer (pH 7.4, 0.5 mL) and EtOH (0.5 mL), or 1/15 M phosphate buffer (pH 7.4, 1.0 mL) was added to a compound in a vial. The suspension was then shaken for 48 h at 25 °C, and undissolved material was separated by filtration. m-Cresol was added as an internal standard (final concentration: 0.05 mg/mL) and the mixture was diluted in DMF and injected onto the HPLC column. The peak area ratio of the sample to the standard was recorded by UV detection at 254 nm. The concentration of the sample solution was calculated using a previously determined calibration curve, corrected for the dilution factor of the sample.

4.5. Molecular modeling

Docking calculations for compound 11d were performed using a similar protocol in our previous research¹⁶ based on the crystal structure of the KSP-inhibitor complex (PDB ID: 3ZCW).²⁷ The protonation states of the amino acid residues of KSP and the direction of the hydrogen atoms involved in the hydrogen bonds were assigned using the Protonate3D algorithm²⁸ implemented in MOE.²⁹ The α4/α6 allosteric site was chosen from the binding sites detected by the MOE-SiteFinder module. Docking pose generation was performed applying pharmacophore restraint to form hydrogen bonds with Asn271 and Leu292. The 100 initial docked candidate poses were optimized by the MMFF94x forcefield³⁰ and the pose with the lowest binding energy (Eₘᵦᵣₚ) estimated by the MM/GBVI method³¹ was adopted as
the predicted binding mode.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.04.008.
References and notes

26 The incompatible results between the in vitro KSP inhibition and cytotoxicity could be attributed to the varied cell membrane permeability. The less solubility of compound 13 in aqueous solution may be disadvantageous to the in vivo studies.
Figure 1. Structures of carbazole- and carboline-type (1–4) and diaryl amine-type (5, 6) KSP inhibitors.

Figure 2. Design of novel KSP inhibitors 7, 8 with diaryl amine scaffolds.

Figure 3. Strategy for the structure–activity relationship study of diaryl amine-type KSP inhibitors.
Figure 4. Design of novel diaryl amine-type KSP inhibitors 13 and 14.

Figure 5. Plausible binding mode of diphenylamine 11d at the interface of helices α4 and α6.
Scheme 1. Synthesis of diaryl amine derivatives. **Reagents and conditions:** (a) Pd$_2$(dba)$_3$, biaryl phosphine ligand, NaOt-Bu, toluene, 100 °C; (b) KOt-Bu, DMF, 40 °C; (c) CuI, ethylene glycol, K$_2$CO$_3$, 2-propanol, 80 °C; (d) Pd/C, HCO$_2$NH$_4$, EtOH, reflux; (e) KOCN, AcOH, H$_2$O, rt; (f) BBr$_3$, CH$_2$Cl$_2$, rt; (g) Zn, AcOH, rt; (h) LiOH·H$_2$O, MeOH, H$_2$O, 50 °C; (i) Lawesson’s reagent, toluene, reflux; (j) Pd(OAc)$_2$, O$_2$, AcOH, 115 °C.
Table 1. KSP inhibitory activities and thermodynamic aqueous solubility of diaryl amines with a pyridine ring and the related compounds.

<table>
<thead>
<tr>
<th>compound</th>
<th>KSP ATPase IC₅₀ (μM)ᵃᵇ</th>
<th>50% EtOH (mg/mL)</th>
<th>solubility phosphate buffer (pH 7.4) (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>¹</td>
<td>0.21</td>
<td>0.424</td>
<td>&lt;1</td>
</tr>
<tr>
<td>3a</td>
<td>0.052</td>
<td>0.472</td>
<td>&lt;1</td>
</tr>
<tr>
<td>3b</td>
<td>0.095</td>
<td>1.76</td>
<td>3.16</td>
</tr>
<tr>
<td>⁷a</td>
<td>&gt;6.3ᵈ</td>
<td>14.3</td>
<td>10.8</td>
</tr>
<tr>
<td>⁷b</td>
<td>&gt;6.3ᵈ</td>
<td>24.0</td>
<td>264</td>
</tr>
</tbody>
</table>

ᵃInhibition of microtubule-activated KSP ATPase activity. ᵇIC₅₀ values were derived from the dose-response curves generated from triplicate data points. ³Solubility in an equal volume of EtOH and 1/15 M phosphate buffer (pH 7.4). ᵈIC₅₀ was ≈7.0 μM.
Table 2. KSP inhibitory activities of diphenylamines with a nitro, amino or urea group and the related carbazoles.

<table>
<thead>
<tr>
<th>compound</th>
<th>R</th>
<th>KSP ATPase IC₅₀ (µM)ᵃᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>R[H,NH₂]CF₃</td>
<td>4b</td>
<td>NH₂</td>
</tr>
<tr>
<td></td>
<td>4c</td>
<td>NHCONH₂</td>
</tr>
<tr>
<td>R[H,NO₂]CF₃</td>
<td>4d</td>
<td>NO₂</td>
</tr>
<tr>
<td></td>
<td>4e</td>
<td>NH₂</td>
</tr>
<tr>
<td></td>
<td>4f</td>
<td>NHCONH₂</td>
</tr>
<tr>
<td>R[NH₂]CF₃</td>
<td>8a</td>
<td>NO₂</td>
</tr>
<tr>
<td></td>
<td>8b</td>
<td>NH₂</td>
</tr>
<tr>
<td></td>
<td>8c</td>
<td>NHCONH₂</td>
</tr>
<tr>
<td>R[NH₂]CF₃</td>
<td>8d</td>
<td>NO₂</td>
</tr>
<tr>
<td></td>
<td>8e</td>
<td>NH₂</td>
</tr>
<tr>
<td></td>
<td>8f</td>
<td>NHCONH₂</td>
</tr>
</tbody>
</table>

ᵃInhibition of microtubule-activated KSP ATPase activity. ᵃᵇIC₅₀ values were derived from the dose-response curves generated from triplicate data points.
Table 3. KSP inhibitory activities of dihydroquinolinone derivatives.

<table>
<thead>
<tr>
<th>compound</th>
<th>X</th>
<th>IC50 (μM)(^{a,b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>5a</td>
<td>NH</td>
<td>0.045</td>
</tr>
<tr>
<td>17a</td>
<td>O</td>
<td>2.0</td>
</tr>
<tr>
<td>20a</td>
<td>S</td>
<td>&gt;6.3</td>
</tr>
<tr>
<td>5b</td>
<td>NH</td>
<td>0.33</td>
</tr>
<tr>
<td>17b</td>
<td>O</td>
<td>&gt;6.3</td>
</tr>
<tr>
<td>20b</td>
<td>S</td>
<td>&gt;6.3</td>
</tr>
<tr>
<td>5c</td>
<td></td>
<td>&gt;6.3</td>
</tr>
</tbody>
</table>

\(^{a}\)Inhibition of microtubule-activated KSP ATPase activity. \(^{b}\)IC50 values were derived from the dose–response curves generated from triplicate data points.
Table 4. KSP inhibitory activities of diphenylamines with a 3-substituent or 3,5-substituents on the right-hand phenyl group.

![Chemical structure of diphenylamines](image)

<table>
<thead>
<tr>
<th>R</th>
<th>KSP ATPase IC₅₀ (µM)ᵃᵇ</th>
<th>R</th>
<th>KSP ATPase IC₅₀ (µM)ᵃᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-CF₃</td>
<td>5a 0.045</td>
<td>3-OMe</td>
<td>11h &gt;6.3</td>
</tr>
<tr>
<td>H</td>
<td>11a &gt;6.3</td>
<td>3-OCF₃</td>
<td>11i 1.2</td>
</tr>
<tr>
<td>3-Et</td>
<td>11b 0.81</td>
<td>3-OH</td>
<td>11j &gt;6.3</td>
</tr>
<tr>
<td>3-i-Pr</td>
<td>11c 0.43</td>
<td>3-NO₂</td>
<td>11k 0.44</td>
</tr>
<tr>
<td>3-t-Bu</td>
<td>11d 0.16</td>
<td>3-NH₂</td>
<td>11l &gt;6.3</td>
</tr>
<tr>
<td>3,5-di-CF₃</td>
<td>11e &gt;6.3</td>
<td>3-CO₂Me</td>
<td>11m &gt;6.3</td>
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<tr>
<td>3-Ph</td>
<td>11f &gt;6.3</td>
<td>3-CO₂H</td>
<td>11n &gt;6.3</td>
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<tr>
<td>3-OPh</td>
<td>11g &gt;6.3</td>
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</tr>
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</table>

ᵃInhibition of microtubule-activated KSP ATPase activity. ᵇIC₅₀ values were derived from the dose–response curves generated from triplicate data points.
Table 5. KSP inhibitory activities of diphenylamines with a heterocycle on the left-hand phenyl group.

<table>
<thead>
<tr>
<th>Ar</th>
<th>KSP ATPase IC₅₀ (μM)ᵃᵇ</th>
<th>Ar</th>
<th>KSP ATPase IC₅₀ (μM)ᵃᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>5a</td>
<td>0.045</td>
<td>12h</td>
<td>0.29</td>
</tr>
<tr>
<td>12a</td>
<td>&gt;6.3</td>
<td>12i</td>
<td>0.051</td>
</tr>
<tr>
<td>12b</td>
<td>&gt;6.3</td>
<td>12j</td>
<td>&gt;6.3</td>
</tr>
<tr>
<td>12c</td>
<td>4.6</td>
<td>12k</td>
<td>0.19</td>
</tr>
<tr>
<td>12d</td>
<td>&gt;6.3</td>
<td>12l</td>
<td>0.050</td>
</tr>
<tr>
<td>12e</td>
<td>3.6</td>
<td>12m</td>
<td>0.43</td>
</tr>
<tr>
<td>12f</td>
<td>&gt;6.3</td>
<td>12n</td>
<td>0.92</td>
</tr>
<tr>
<td>12g</td>
<td>0.81</td>
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</tbody>
</table>

ᵃInhibition of microtubule-activated KSP ATPase activity. ᵇIC₅₀ values were derived from the dose–response curves generated from triplicate data points.
Table 6. KSP inhibitory activities and physicochemical properties of diaryl amine derivatives 5a, 6, 12i, 13 and 14.

<table>
<thead>
<tr>
<th></th>
<th>5a</th>
<th>12i</th>
<th>12l</th>
</tr>
</thead>
<tbody>
<tr>
<td>KSP ATPase IC(_{50}) ((\mu)M)(^{a,b})</td>
<td>0.045</td>
<td>0.051</td>
<td>0.050</td>
</tr>
<tr>
<td>Solubility in 50% EtOH (mg/mL)(^c)</td>
<td>1.80</td>
<td>1.70</td>
<td>7.39</td>
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<tr>
<td>Solubility in phosphate buffer (pH 7.4) ((\mu)g/mL)</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>melting point ((^\circ)C)</td>
<td>190</td>
<td>166</td>
<td>140</td>
</tr>
<tr>
<td>ClogP(^d)</td>
<td>4.2</td>
<td>4.0</td>
<td>4.4</td>
</tr>
<tr>
<td>HPLC retention time (min)(^e)</td>
<td>24.4</td>
<td>28.2</td>
<td>27.0</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>6</th>
<th>13</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>KSP ATPase IC(_{50}) ((\mu)M)(^{a,b})</td>
<td>0.068</td>
<td>0.035</td>
<td>0.050</td>
</tr>
<tr>
<td>Solubility in 50% EtOH (mg/mL)(^c)</td>
<td>3.51</td>
<td>0.669</td>
<td>4.82</td>
</tr>
<tr>
<td>Solubility in phosphate buffer (pH 7.4) ((\mu)g/mL)</td>
<td>6.12</td>
<td>1.11</td>
<td>8.07</td>
</tr>
<tr>
<td>melting point ((^\circ)C)</td>
<td>177</td>
<td>217</td>
<td>185</td>
</tr>
<tr>
<td>ClogP(^d)</td>
<td>3.4</td>
<td>3.2</td>
<td>3.6</td>
</tr>
<tr>
<td>HPLC retention time (min)(^e)</td>
<td>7.0</td>
<td>13.8</td>
<td>10.8</td>
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</table>

\(^a\)Inhibition of microtubule-activated KSP ATPase activity. \(^b\)IC\(_{50}\) values were derived from the dose–response curves generated from triplicate data points. \(^c\)Solubility in 50% EtOH [an equal volume of EtOH and 1/15 M phosphate buffer (pH 7.4)]. \(^d\)CLogP values were calculated with ChemBioDraw Ultra 12.0. \(^e\)HPLC analysis was carried out on a Cosmosil 5C18-ARII column (4.6 \(\times\) 250 mm) and the material eluted by a linear MeCN gradient (30-70% over 40 min) in 0.1% TFA; flow rate of 1 mL/min.
Table 7. Inhibitory effects on cell proliferation of diaryl amine-type KSP inhibitors toward A549, HCT-116 and MCF-7.

<table>
<thead>
<tr>
<th>compound</th>
<th>IC50 (μM)a</th>
<th>A549</th>
<th>HCT-116</th>
<th>MCF-7</th>
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<tbody>
<tr>
<td>5a</td>
<td>4.2</td>
<td>8.9</td>
<td>11</td>
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<td>6</td>
<td>2.5</td>
<td>4.4</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td>12i</td>
<td>4.1</td>
<td>6.8</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>12l</td>
<td>4.5</td>
<td>5.7</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>1.5</td>
<td>2.6</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>5.0</td>
<td>6.5</td>
<td>9.1</td>
<td></td>
</tr>
</tbody>
</table>

aIC50 values were derived from the dose–response curves generated from triplicate data points.