Design, Synthesis and Evaluation of Tetrahydroisoquinolines as New Kinesin Spindle Protein Inhibitors

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In this study, a series of tetrahydroisoquinolines have been synthesized and identified as novel kinesine spindle protein (KSP) inhibitors based on the pharmacophore we have mapped and the crystal structure of monastrol bound to the target protein. The KSP inhibitory activities of all the designed compounds were tested using cloned Human KSP protein. All thirteen compounds were more potent than the control, monastrol, in Human KSP protein adenosine triphosphatase (ATPase) assays. Three compounds (1b, 1g, 1h) exhibited over 100 times higher potency than monastrol. Cytotoxic results *in vitro* by MTT method indicated that nine of these compounds (1a, 1b, 1c, 1d, 1e, 1g, 1h, 1j, 1k) were more active than monastrol. In particular, compounds 1b and 1g, each of which contains a hydrophilic group on the side chain at the 2-position, exhibited excellent cell-killing activities against HepG2 cells.

Key words kinesin spindle protein inhibitor; tetrahydroisoquinoline; pharmacophore; anticancer

Kinesin spindle protein (KSP), also known as *Hs* Eg5, is a member of the kinesin superfamily of molecular motors that utilize the energy generated from the hydrolysis of ATP to transport vesicles, organelles, and microtubules.[1)] It is also required for centrosome separation during prophase or prometaphase.[2)] Inhibition of KSP can prevent the formation of normal bipolar spindle which leads to mitotic arrest with a characteristic monoastral phenotype, followed by apoptosis in the transformed cells without disturbing the microtubules.[3,4)] Therefore, KSP inhibition represents a novel and specific mechanism for targeting the mitotic spindle without any neuropathy-associated, mechanism-based side effects which are quite common for taxanes and other microtubule-targeting natural products. In recent years, several classes of KSP inhibitors have been reported as novel anti-cancer agents.[5—23)]

Based on the KSP inhibitors reported in the literature, we have identified the pharmacophore and performed binding mode analysis for KSP inhibitors.[24,25)] In our previous work, we identified the pharmacophore of KSP inhibitors which consists of four chemical features (one hydrogen-bond acceptor, one hydrogen-bond donor, one aromatic ring, and one hydrophobic group).[24)] After mapping the pharmacophore with several classes of KSP inhibitors, we found that most KSP inhibitors contained four parts: an aromatic ring (part 1), a hydrophobic group (part 2), a side chain (part 3) and a flexible heterocyclic ring (part 4) which connected the other three components (Fig. 1). Moreover, the hydrogen-bond acceptor or hydrogen-bond donor at the side chain can improve KSP inhibitory activity to a certain extent. In 2004, the crystal structure of monastrol bound to KSP, in complex with ADP, was reported by Yan *et al.*[26)] This binding mode of KSP inhibitors has been studied extensively, offering us a good opportunity to discover novel KSP inhibitors as anti-cancer agents.

Molecular Design As shown in Fig. 2, monastrol contains four parts of the pharmacophore, although the hydrophobic group of part 2 is not obvious. *R*-Mon-97 (IC_{50}= 150 nM),[27)] in which there is a hydrophobic group of part 2 but without the side chain of part 3, is sixty times more potent in inhibiting KSP activity than monastrol. This phenomenon suggests that the hydrophobic group of part 2 is essential for inhibiting KSP activity. *S*-Mon-97 (IC_{50}=650 nM),[27)]

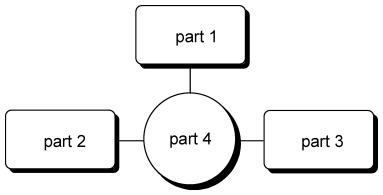

Fig. 1. The Four Parts of an Active KSP Inhibitor in Our Model (Part 1: Aromatic Ring; Part 2: Hydrophobic Group; Part 3: Side Chain; Part 4: Flexible Heterocyclic Ring)

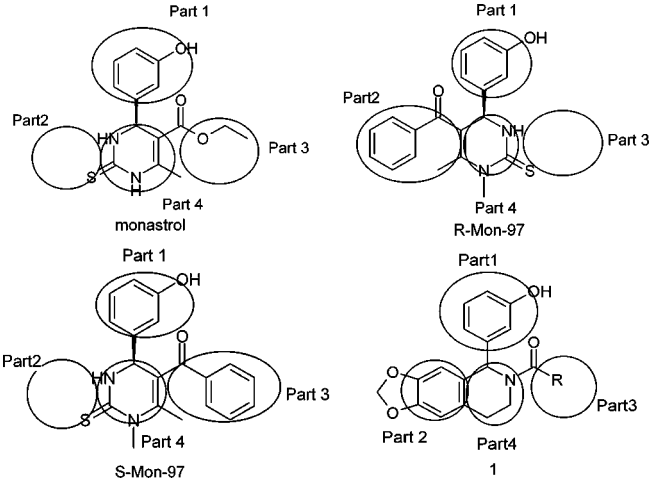

Fig. 2. Design of New KSP Inhibitors (**1**) Based on the Pharmacophore

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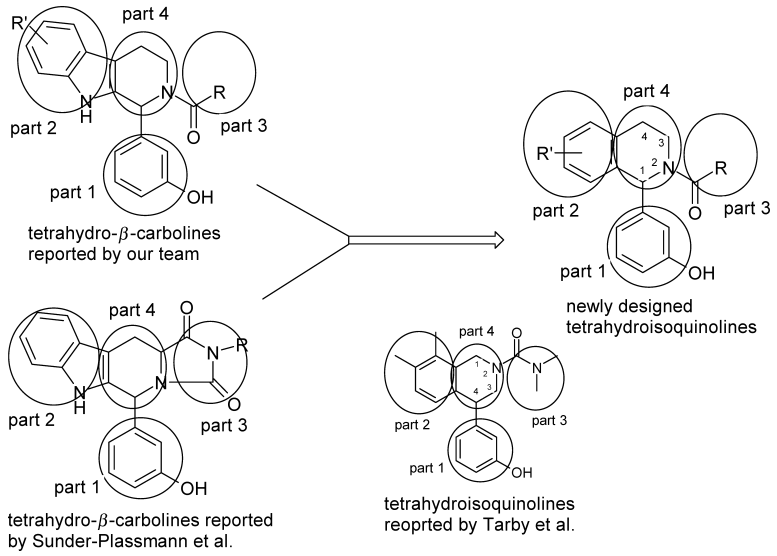


Fig. 3. Tetrahydroisoquinoline Is Selected as the Core of New KSP Inhibitors

which does not have a hydrophobic group of part 2 but possesses an aryl group in the side chain of part 3, is also sixteen times more potent than monastrol. This suggests that the introduction of aryl group to part 3 may increase the KSP inhibitory activity.

Based on this pharmacophore, we identified and analyzed some representative KSP inhibitors. A novel series of KSP inhibitors were then designed (**1** in Fig. 2). Part 1 of the newly designed KSP inhibitors is the same as that of monastrol, *S*-Mon-97, and *R*-Mon-97, which is generally considered to be essential for potent KSP inhibitory activity. In several patents,[28,29] a side chain was attached to the N atom of a six-ring core which may be used to replace part 4. Our team have reported a series of tetrahydro-β-carbolines as KSP inhibitors recently,[30,31] which are different from those reported by Sunder-Plassmann *et al.*[32] It could be found that the tetrahydro-β-carboline core is shared by part 2 and part 4 of our pharmacophore. Benzene ring is a typical hydrophobic group, thus a phenyl group could be introduced to fit part 2 of our pharmacophore. After changing the indole group of tetrahydro-β-carboline core into phenyl group, a tetrahydroisoquinoline core could be gained (as shown in Fig. 3). Although, recently, a series of 4-phenyl tetrahydroisoquinolines were reported as potent KSP inhibitors,[14] in which there is an aryl group connected to the C4 atom of the reported tetrahydroisoquinolines, in our newly designed compounds, a 3′-hydroxyphenyl group was attached to C1 position of tetrahydroisoquinolines. In the structure of these 1-(3′-hydroxyphenyl)-tetrahydroisoquinoline compounds, the tetrahydroisoquinoline ring is shared by part 2 and part 4 of KSP inhibitor **1**. To facilitate the synthesis, a methylenedioxy substitution was introduced to the tetrahydroisoquinoline core. Combining these fragments, an aromatic ring (part 1), a hydrophobic group (part 2), a side chain (part 3) and a flexible heterocyclic ring (part 4), as shown in Fig. 2, are all present in the newly designed compounds.

In order to optimize the design of KSP inhibitor **1**, the binding model of compound **1** with KSP was analyzed and compared with the crystal structure of monastrol bound to KSP. As shown in Fig. 4, the benzene ring of Tyr211 and the

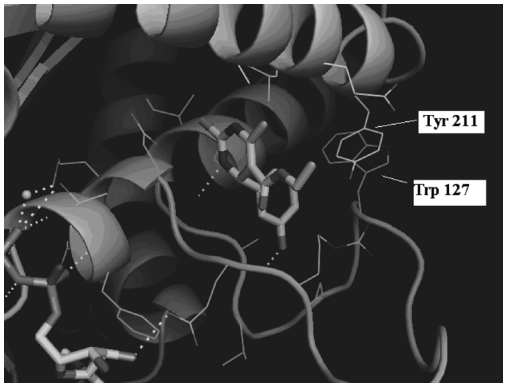


Fig. 4. The Binding Site of Monastrol with KSP

indole ring of Trp127 were close to the side chain of monastrol, which may explain why an aryl group in part 3 can increase the binding affinity to KSP. From the crystal structure, it is clear that the side chain of part 3 is exposed to the solvent, as was reported in previous literature.[10,12] Thus, a hydrophilic group such as an amino group at this location may also increase the KSP inhibitory activity. Taken into consideration all the information, a series of molecules were designed which are listed in Table 1.

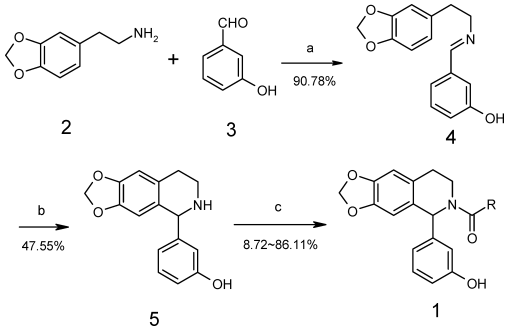
Chemistry The synthesis of the tetrahydroisoquinoline-based compounds is presented in Chart 1. Condensation of piperoethylamine (**2**) with 3-hydroxybenzaldehyde (**3**), followed by cyclization in the presence of HCl, gave the intermediate **5**. In the last step, acylation with different acids led to thirteen desired compounds which were confirmed by IR, ^{1}H-NMR, and elemental analysis.

KSP ATPase Assay The motor and linker domain of human KSP protein was cloned, expressed in *Escherichia coli* BL21 (DE3) cells and then purified. The effects of temperature, pH, metal ions and dimethyl sulfoxide (DMSO) on ATPase activity were investigated to validate the assay. KSP inhibitory activity was assessed by measuring the release of inorganic phosphate from ATP hydrolysis through absorbance detection of a malachite green–phosphate com-

Table 1. Bioactivity of the Synthesized KSP Inhibitors

Compounds	R	KSP IC_{50} (μM)	Cytotoxicity[a] (μM)
1a	$-CH_2CH_2CH_3$	1.09	12.65
1b	$-CH_2CH_2NH_2$	0.02	8.34
1c	$-CH_3$	0.51	15.44
1d	$PhCH_2-$	1.02	13.01
1e	$-OCH_2CH_3$	0.17	10.33
1f	$-OCH_3$	4.66	21.41
1g	4-OH–Ph–	0.04	6.08
1h	$4\text{-}CH_3\text{–Ph–}$	0.06	10.97
1i	Naphth–CH_2–	0.23	25.33
1j	$4\text{-}OCH_3\text{–Ph–}$	0.95	19.22
1k	4-Cl–Ph–	0.35	14.21
1l	3-Cl–Ph–	3.65	29.92
1m	$4\text{-}NO_2\text{–}PhCH_2\text{–}$	7.14	36.38
Monastrol	—	7.45	20.45

a) Presented as IC_{50} values against HepG2 cells.



Reaction conditions: (a) EtOH, room temperature; (b) 24% HCl, 60 °C; (c) RCOOH, PyBOP, TEA, dry THF.

Chart 1. General Route for the Synthesis of Tetrahydroisoquinolines

plex.[33] The IC_{50} values of the target compounds against KSP were determined by measuring the activities of microtubule-activated ATPase using monastrol as the control.

As shown in Table 1, all thirteen compounds were more potent than monastrol in inhibiting KSP activity. Compound **1b**, **1g** and **1h**, with the side chains being aminopropionyl, 4′-hydroxybenzoyl and 4′-methylbenzoyl respectively, were over 100 times more potent than monastrol. This finding proved that tetrahydroisoquinolines with a 3′-hydroxyphenyl group attached to C1 are potent KSP inhibitors. It was also found that an aryl group attached to N2, as well as a hydrophilic group at the end of the side chain attached to N2, could increase the KSP inhibitory activity of the compound. Taken together, these results confirm the hypothesis we proposed during the pharmacophore design.

Cytotoxic Activity *in Vitro* All the tetrahydroisoquinolines were evaluated for cytotoxicity to human liver cancer HepG2 cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. As shown in Table 1, nine compounds (**1a**, **1b**, **1c**, **1d**, **1e**, **1g**, **1h**, **1j**, **1k**) exhibited more potent growth inhibition of HepG2 than monastrol. In particular, compounds **1b** and **1g**, with the side chains being aminopropionyl and 4′-hydroxybenzoyl respectively, exhibited excellent cell-killing activities against HepG2 cells. Interestingly, there is a hydrophilic group at the end of the side chain of part 3 in each of the two structures, which suggested that the terminal –OH or $-NH_2$ of the side chain may contribute to cytotoxicity against cells. Overall, the result from the cytotoxicity assay has good correlation with the KSP inhibitory activities.

Conclusion

In summary, we report the design, synthesis, and evaluation of a series of tetrahydroisoquinolines derivatives (**1a**—**m**) as novel KSP inhibitors based on the crystal structure of monastrol bound to KSP protein, as well as pharmacophore analysis based on the available literature. Generally, all thirteen synthesized compounds were more potent than monastrol in human KSP protein ATPase assays. Three KSP inhibitors (**1b**, **1g**, **1h**) had over 100 times higher potency than monastrol. *In vitro* cytotoxicity assay using the MTT method indicated that nine compounds (**1a**, **1b**, **1c**, **1d**, **1e**, **1g**, **1h**, **1j**, **1k**) were more cytotoxic than monastrol. Compounds **1b** and **1g**, both containing a hydrophilic group at the end of the side chain at 2-position, exhibited excellent cell-killing activities against HepG2. This discovery may yield novel lead compounds for further modification/optimization in the development of potent KSP inhibitors as anti-cancer agents.

Experimental

Chemistry All chemicals were obtained commercially and used without further purification. Melting points were determined on Mel-Temp II apparatus. Infrared spectra were acquired on Nicolet Impact 410 spectrophotometer using a KBr film. The absorption band is given in cm^{-1}. ^{1}H-NMR spectra were recorded on a Bruker ACF-300 spectrometer (300 MHz). Chemical shifts are presented in ppm relative to tetramethylsilane. Mass spectra were obtained on a Mariner Mass Spectrum, a GC-2010 mass spectrometer, or a MAT-212 mass spectrometer. Elemental analyses were determined on a Carlo Erba 1106 elementary analysis apparatus.

3-[(2-(3,4-Dioxolo)phenylethylimine)methyl]phenol (4) Piperoethylamine **2** (10.00 g, 0.06 mol) was added into a solution of 3-hydroxybenzaldehyde **3** (7.40 g, 0.06 mol) in ethanol (60 ml) at room temperature. The mixture was stirred for 10 min and cooled. The precipitate (**4**) was collected by filtration as an off-white powder (14.80 g, yield 90.8%): mp 153—155 °C. IR (cm^{-1}): 3474, 3050, 2906, 2867, 1649, 1595, 1502, 1454, 1273, 1247, 1038, 923, 775, 686. ^{1}H-NMR ($CDCl_3$, ppm) δ: 2.83 (t, J=7.2 Hz, 2H, –C<u>H</u>$_2$CH$_2$N), 3.74 (t, J=7.2 Hz, 2H, –CH$_2$C<u>H</u>$_2$N), 5.95 (s, 2H, –OC<u>H</u>$_2$O–), 6.68—7.25 (m, 7H, Ar-H), 8.17 (s, 1H, N=CH–), 9.54 (s, 1H, –OH). EI-MS: 269 [M].

1-(3′-Hydroxyphenyl)-6,7-dioxolo-1,2,3,4-tetrahydroisoquinoline (5) The mixture of **4** (10.00 g, 0.037 mol) and HCl (24%, 50 ml) was heated to 60 °C under N_2. Eight hours later, the mixture was cooled and filtered. The solid was washed with acetone to yield the title compound as an off-white powder (5.40 g, yield 47.6%): mp 213—215 °C. IR (cm^{-1}): 3423, 3224, 2934, 2769, 1589, 1503, 1486, 1243, 1039, 938, 787, 700. ^{1}H-NMR (DMSO-d_6, ppm) δ: 2.88—2.99 (m, 2H, –C<u>H</u>$_2$CH$_2$N), 3.13—3.20 (m, 2H, –CH$_2$C<u>H</u>$_2$N), 5.31 (s, 1H, N<u>H</u>), 5.93 (s, 2H, –OC<u>H</u>$_2$O–), 6.19 (s, 1H, C<u>H</u>), 6.68—7.19 (m, 6H, Ar-H), 9.55 (s, 1H, –O<u>H</u>). EI-MS: 269 [M].

General Procedure for the Synthesis of the Target Compounds 1a, 1c—m A mixture of the proper organic acid (0.98 mmol), PyBOP (0.51 g, 0.98 mmol), TEA (0.3 ml) and dry THF (15 ml) was stirred at room temperature for 10 min. Compound **5** (0.30 g, 0.98 mmol) was then added. The mixture was stirred for 3 h and filtered. The filtrate was purified by column chromatography (petroleum ether/ethyl acetate=2/1).

1-(3′-Hydroxyphenyl)-2-butyryl-6,7-dioxolo-1,2,3,4-tetrahydroisoquinoline (1a) White powder, yield 57.1%; mp 159—160 °C. IR (cm^{-1}): 3192, 2957, 2873, 1593, 1485, 1275, 1231, 1038, 925, 774, 706. ^{1}H-NMR (DMSO-d_6, ppm) δ: 0.87 (t, J=7.37 Hz, 3H, –CH$_2$CH$_2$C<u>H</u>$_3$), 1.53 (m, 2H, –CH$_2$C<u>H</u>$_2$CH$_3$), 2.33 (m, 2H, –C<u>H</u>$_2$CH$_2$N), 2.6 (m, 2H, –C<u>H</u>$_2$CH$_2$CH$_3$), 2.61—2.81 (m, 2H, –CH$_2$C<u>H</u>$_2$N), 5.95 (s, 2H, –OC<u>H</u>$_2$O–), 6.68 (s, 1H, C<u>H</u>), 6.49—6.60, 6.76—7.09 (m, 6H, Ar-H), 9.24 (s, 1H, –O<u>H</u>). EI-MS: 339 [M]. *Anal.* Calcd for $C_{20}H_{21}NO_4$: C, 70.78; H, 6.24; N, 4.13. Found: C, 70.78; H, 6.24; N, 4.00.

1-(3′-Hydroxyphenyl)-2-acetyl-6,7-dioxolo-1,2,3,4-tetrahydroisoquinoline (1c) White powder, yield 55.7%; mp 198—200 °C. IR (cm^{-1}): 3265, 2903, 1618, 1584, 1484, 1448, 1235, 1037, 921, 883, 775. ^{1}H-NMR

(DMSO-d_6, ppm) δ: 2.09 (s, 3H, –COC$\underline{H}_3$), 2.64—2.85 (m, 2H, –C$\underline{H}_2$CH$_2$N), 3.31—3.70 (m, 2H, –CH$_2$C$\underline{H}_2$N), 5.98 (s, 2H, –OC$\underline{H}_2$O–), 6.71 (s, 1H, C$\underline{H}$), 6.52—6.63, 6.77—7.12 (m, 6H, Ar-H), 9.28 (s, 1H, –O$\underline{H}$). EI-MS: 311 [M]. *Anal.* Calcd for $C_{18}H_{17}NO_4$: C, 69.44; H, 5.50; N, 4.50. Found: C, 69.48; H, 5.52; N, 4.54.

1-(3′-Hydroxyphenyl)-2-phenylacetyl-6,7-dioxolo-1,2,3,4-tetrahydroisoquinoline (1d) White powder, yield 21.1%; mp 184—185 °C. IR (cm^{-1}): 3098, 2895, 1590, 1481, 1275, 1229, 1038, 921, 742, 692. ^{1}H-NMR (DMSO-d_6, ppm) δ: 2.60 (s, 2H, –C$\underline{H}_2$CO), *ca.* 2.6 (m, 2H, –C$\underline{H}_2$CH$_2$N), 3.81 (m, 2H, –CH$_2$C$\underline{H}_2$N), 5.97 (s, 2H, –OC$\underline{H}_2$O–), 6.68 (s, 1H, C$\underline{H}$), 6.53—6.62, 6.75—7.30 (m, 11H, Ar-H), 9.29 (s, 1H, –O$\underline{H}$). EI-MS: 387 [M]. *Anal.* Calcd for $C_{24}H_{21}NO_4$: C, 74.40; H, 5.46; N, 3.62. Found: C, 74.40; H, 5.47; N, 3.54.

1-(3′-Hydroxyphenyl)-2-ethoxycarboxyl-6,7-dioxolo-1,2,3,4-tetrahydroisoquinoline (1e) White powder, yield 44.8%; mp 180—181 °C. IR (cm^{-1}): 3255, 2973, 2877, 1660, 1596, 1487, 1438, 1249, 1111, 928, 775. ^{1}H-NMR (DMSO-d_6, ppm) δ: 1.26 (m, 3H, –CH$_2$C$\underline{H}_3$), 2.61—2.89 (m, 2H, –C$\underline{H}_2$CH$_2$N), 3.16—3.22 (m, 2H, –CH$_2$C$\underline{H}_2$N), 4.18 (m, 2H, –C$\underline{H}_2$CH$_3$), 5.92 (s, 2H, –OC$\underline{H}_2$O–), 6.63 (s, 1H, C$\underline{H}$), 6.49, 6.71—7.16 (m, 6H, Ar-H). EI-MS: 341 [M]. *Anal.* Calcd for $C_{19}H_{19}NO_5$: C, 66.85; H, 5.61; N, 4.10. Found: C, 66.73; H, 5.70; N, 4.03.

1-(3′-Hydroxyphenyl)-2-methoxycarboxyl-6,7-dioxolo-1,2,3,4-tetrahydroisoquinoline (1f) White powder, yield 49.8%; mp 244—246 °C. IR (cm^{-1}): 3263, 2904, 1658, 1594, 1487, 1448, 1246, 1217, 1039, 942, 927, 776, 700. ^{1}H-NMR ($CDCl_3$, ppm) δ: 2.62—2.80 (m, 2H, –C$\underline{H}_2$CH$_2$N), 3.20—3.50 (m, 2H, –CH$_2$C$\underline{H}_2$N), 3.76 (s, 3H, –C$\underline{H}_3$), 5.91 (s, 2H, –OC$\underline{H}_2$O–), 6.63 (s, 1H, C$\underline{H}$), 6.50, 6.71—7.29 (m, 6H, Ar-H). EI-MS: 327 [M]. *Anal.* Calcd for $C_{18}H_{17}NO_5$: C, 66.05; H, 5.23; N, 4.28. Found: C, 65.92; H, 5.36; N, 4.16.

1-(3′-Hydroxyphenyl)-2-(4′-hydroxyphenylacetyl)-6,7-dioxolo-1,2,3,4-tetrahydroisoquinoline (1g) White powder, yield 55.0%; mp 140 °C. IR (cm^{-1}): 3308, 2954, 1734, 1562, 1484, 1233, 1037, 939, 778. ^{1}H-NMR (DMSO-d_6, ppm) δ: 2.63—2.89 (m, 2H, –C$\underline{H}_2$CH$_2$N), 3.21—3.60 (m, 2H, –CH$_2$C$\underline{H}_2$N), 5.98 (s, 2H, –OC$\underline{H}_2$O–), 6.97 (s, 1H, C$\underline{H}$), 6.63—6.65, 6.79—7.23 (m, 10H, Ar-H), 9.34 (s, 1H, –O$\underline{H}$), 9.81 (s, 1H, –O$\underline{H}$). EI-MS: 389 [M]. *Anal.* Calcd for $C_{23}H_{19}NO_5.C_2H_5OH$: C, 68.96; H, 5.75; N, 3.19. Found: C, 68.68; H, 5.88; N, 2.90.

1-(3′-Hydroxyphenyl)-2-(4′-methylbenzoyl)-6,7-dioxolo-1,2,3,4-tetrahydroisoquinoline (1h) White powder, yield 31.6%; mp 186—187 °C. IR (cm^{-1}): 3414, 3170, 2886, 1594, 1440, 1235, 1033, 932, 829, 781, 706. ^{1}H-NMR (DMSO-d_6, ppm) δ: 2.34 (s, 3H, –C$\underline{H}_3$), 2.60—2.85 (m, 2H, –C$\underline{H}_2$CH$_2$N), 3.20—3.48 (m, 2H, –CH$_2$C$\underline{H}_2$N), 5.99 (s, 2H, –OC$\underline{H}_2$O–), 6.79 (s, 1H, C$\underline{H}$), 6.45—6.71, 7.12—7.26 (m, 10H, Ar-H), 9.38 (s, 1H, –O$\underline{H}$). EI-MS: 387 [M]. *Anal.* Calcd for $C_{24}H_{21}NO_4$: C, 74.40; H, 5.46; N, 3.62. Found: C, 74.47; H, 5.53; N, 3.51.

1-(3′-Hydroxyphenyl)-2-(β-naphthalin acetyl)-6,7-dioxolo-1,2,3,4- tetrahydroisoquinoline (1i) White powder, yield 14.0%; mp 224—226 °C. IR (cm^{-1}): 3234, 2890, 1591, 1502, 1452, 1238, 1042, 925, 790, 703. ^{1}H-NMR (DMSO-d_6, ppm) δ: 2.64—2.85 (m, 2H, –C$\underline{H}_2$CH$_2$N), 3.29—3.45 (m, 2H, –CH$_2$C$\underline{H}_2$N), 4.25 (s, 2H, –C$\underline{H}_2$CO), 5.97 (s, 2H, –OC$\underline{H}_2$O–), 6.79 (s, 1H, C$\underline{H}$), 6.57—6.71, 7.08—7.94 (m, 13H, Ar-H), 9.30 (s, 1H, –O$\underline{H}$). EI-MS: 437 [M]. *Anal.* Calcd for $C_{28}H_{23}NO_4$: C, 76.87; H, 5.30; N, 3.20. Found: C, 76.58; H, 5.46; N, 3.07.

1-(3′-Hydroxyphenyl)-2-(4′-methoxybenzoyl)-6,7-dioxolo-1,2,3,4-tetrahydroisoquinoline (1j) White powder, yield 25.3%; mp 192 °C. IR (cm^{-1}): 3203, 2887, 1589, 1447, 1252, 1176, 1038, 941, 836, 772, 744. ^{1}H-NMR (DMSO-d_6, ppm) δ: 2.63—2.86 (m, 2H, –C$\underline{H}_2$CH$_2$N), 3.22—3.60 (m, 2H, –CH$_2$C$\underline{H}_2$N), 5.99 (s, 2H, –OC$\underline{H}_2$O–), 6.79 (s, 1H, C$\underline{H}$), 6.63—6.70, 6.98—7.97 (m, 10H, Ar-H), 9.34 (s, 1H, –O$\underline{H}$). EI-MS: 403 [M]. *Anal.* Calcd for $C_{24}H_{21}NO_5 \cdot H_2O$: C, 68.40; H, 5.46; N, 3.32. Found: C, 68.32; H, 5.62; N, 2.99.

1-(3′-Hydroxyphenyl)-2-(4′-chlorobenzoyl)-6,7-dioxolo-1,2,3,4-tetrahydroisoquinoline (1k) White powder, yield 25.0%; mp 191—193 °C. IR (cm^{-1}): 3182, 1586, 1484, 1445, 1241, 1039, 941, 845, 771, 700. ^{1}H-NMR (DMSO-d_6, ppm) δ: 2.64—2.90 (m, 2H, –C$\underline{H}_2$CH$_2$N), 3.39—3.50 (m, 2H, –CH$_2$C$\underline{H}_2$N), 6.00 (s, 2H, –OC$\underline{H}_2$O–), 6.74 (s, 1H, C$\underline{H}$), 6.66, 6.81—7.54 (m, 10H, Ar-H), 9.37 (s, 1H, –O$\underline{H}$). EI-MS: 407 [M]. *Anal.* Calcd for $C_{23}H_{18}ClNO_4 \cdot 0.5H_2O$: C, 66.26; H, 4.56; N, 3.36. Found: C, 65.99; H, 4.69; N, 3.23.

1-(3′-Hydroxyphenyl)-2-(3′-chlorobenzoyl)-6,7-dioxolo-1,2,3,4-tetrahydroisoquinoline (1l) White powder, yield 37.5%; mp 91—93 °C. IR (cm^{-1}): 3262, 2895, 1592, 1484, 1445, 1235, 1037, 940, 774, 697. ^{1}H-NMR (DMSO-d_6, ppm) δ: 2.63—2.86 (m, 2H, –C$\underline{H}_2$CH$_2$N), 3.34—3.47 (m, 2H, –CH$_2$C$\underline{H}_2$N), 5.99 (s, 2H, –OC$\underline{H}_2$O–), 6.74 (s, 1H, C$\underline{H}$), 6.65—6.67, 6.80—7.90 (m, 10H, Ar-H), 9.37 (s, 1H, –O$\underline{H}$). EI-MS: 407 [M]. *Anal.* Calcd for $C_{23}H_{18}ClNO_4 \cdot 0.5H_2O$: C, 66.26; H, 4.56; N, 3.36. Found: C, 66.38; H, 4.70; N, 3.26.

1-(3′-Hydroxyphenyl)-2-(4′-nitrophenylacetyl)-6,7-dioxolo-1,2,3,4-tetrahydroisoquinoline (1m) Pale yellow powder, yield 8.7%; mp 153—155 °C. IR (cm^{-1}): 3415, 3079, 2931, 1618, 1519, 1483, 1345, 1236, 1037, 922, 858, 773, 698. ^{1}H-NMR (DMSO-d_6, ppm) δ: 2.66—2.80 (m, 2H, –C$\underline{H}_2$CH$_2$N), 2.67 (s, 2H, –C$\underline{H}_2$CO), 3.34—3.50 (m, 2H, –CH$_2$C$\underline{H}_2$N), 5.93 (s, 2H, –OC$\underline{H}_2$O–), 6.77 (s, 1H, C$\underline{H}$), 6.48—6.73, 6.90—8.14 (m, 10H, Ar-H). EI-MS: 432 [M]. *Anal.* ($C_{24}H_{20}N_2O_6$) C, H, N. Calcd: 66.66, 4.66, 6.48; Found: 66.44, 4.86, 6.41.

1-(3′-Hydroxyphenyl)-2-(Boc-β-alaninyl)-6,7-dioxolo-1,2,3,4-tetrahydroisoquinoline (1b′) Following the synthetic procedure of compound (**1a**), the title compound was obtained from compound **5** (0.50 g, 1.64 mmol), (0.31 g, 1.64 mmol), PyBOP (0.85 g, 1.64 mmol) as a colorless oil (0.62 g, yield 86.1%). IR (cm^{-1}): 3342, 2974, 1686, 1617, 1484, 1453, 1366, 1237, 1167, 1038, 936, 864, 778, 702. ^{1}H-NMR (DMSO-d_6, ppm) δ: 1.41 (s, 9H, $CH_3 \times 3$), 2.52 (m, 2H, –CH$_2$C$\underline{H}_2$CO), 3.45 (m, 4H, –CH$_2$C$\underline{H}_2$N and –CH$_2$C$\underline{H}_2$NHBoc), 5.93 (s, 2H, –OC$\underline{H}_2$O–), 6.73 (s, 1H, C$\underline{H}$), 6.54—6.62, 6.76—7.26 (m, 6H, Ar-H). EI-MS: 440 [M].

1-(3′-Hydroxyphenyl)-2-(β-aminopropionyl)-6,7-dioxolo-1,2,3,4-tetrahydroisoquinoline (1b) Compound **1b′** (0.62 g, 1.40 mmol) was dissolved in ethyl acetate (25 ml), and the solution was saturated by HCl gas. The mixture was stirred at room temperature for 3.5 h and evaporated to dryness. Dry ether (10 ml) was added to the residue and the precipitate was collected by filtration. The title compound was dried in vacuum to give **1b** as a pale yellow powder (0.20 g, yield 37.7%): mp 125 °C (dec.). IR (cm^{-1}): 3435, 3223, 2970, 1614, 1484, 1453, 1236, 1037, 922, 774. ^{1}H-NMR (DMSO-d_6, ppm) δ: 2.67—2.71 (m, 2H, –NCH$_2$C$\underline{H}_2$CO), 2.77—2.86 (m, 2H, –C$\underline{H}_2$CO), 3.01—3.06 (m, 2H, –CH$_2$C$\underline{H}_2$NH$_2$), 3.32—3.45 (m, 2H, –CH$_2$C$\underline{H}_2$N), 5.98 (s, 2H, –OC$\underline{H}_2$O–), 6.74 (s, 1H, C$\underline{H}$), 6.53—6.64, 6.79—7.09 (m, 6H, Ar-H), 7.75 (br, 2H, –N$\underline{H}_2$), 9.34 (s, 1H, –O$\underline{H}$). EI-MS: 340 [M]. *Anal.* Calcd for $C_{19}H_{20}N_2O_4 \cdot HCl \cdot 1.5H_2O$: C, 56.51; H, 5.70; N, 6.94. Found: C, 56.29; H, 5.96; N, 6.98.

Preparation of KSP The coding regions were PCR amplified from a template (obtained in our laboratory) containing full-length human KSP. The primers used were: forward 5′-TAT AGG GCG AAT TCC GCC ATG GCG TCG CAG CCA-3′ and reverse 5′-ACG GGC TGC AGC AAG CTC GAG TTT TAA ACG TTC TAT-3′. The region encoding residues 2—386 was sub-cloned into pET28a (NOVAGEN).[34] Protein expression in *E. coli* cells was induced with 0.5 mM IPTG. Cells were harvested after 20 h of growth at 20 °C and then lysed by sonication. The soluble lysate was clarified by centrifugation and applied to a SP-Sepharose column (Amersham Pharmacia Biotech) in a buffer A (20 mM Na-PIPES, pH 6.3; 20 mM NaCl; 1 mM $MgCl_2$; 1 mM Na-EGTA). Protein was eluted with a linear gradient of 20—1000 mM NaCl. KSP was identified by SDS-PAGE, and then applied to Mono-Q columns (Amersham Pharmacia Biotech) in a buffer B (20 mM Tris–HCl, pH 8.8; 1 mM $MgCl_2$; 1 mM Na-EGTA). A gradient from 0—1000 mM NaCl was used to elute KSP.[35] Fractions were analyzed by SDS-PAGE. The most concentrated fraction was dialyzed against ATPase buffer (20 mM Na-PIPES, pH 7.5; 1 mM $MgCl_2$; 1 mM Na-EGTA) and then aliquoted, frozen in liquid nitrogen, and stored at −80 °C for future use.

ATPase Activity Assay All experiments were carried out at room temperature. The reagents were added to the wells of a 96-well clear plate and the final reaction of the assay contained 20 mM PIPES, pH 7.5, 5.0 mM $MgCl_2$, 1 mM EGTA, 10 μM paclitaxel, 0.6 mM tubulin (MT), 0.5 mM ATP, 2% DMSO containing inhibitors (DMSO had no effect on the ATPase activity as shown later) in a reaction volume of 100 μl. Reactions were started by addition of ATP and the plates were incubated at 37 °C for 30 min. Malachite-green based reagents were then added to detect the release of inorganic phosphate.[36] The plates were incubated for an additional 5 min at room temperature, and 10 μl of 34% sodium citrate was added. The absorbance at 610 nm was determined using Multiskan Spectrum Microplate Spectrophotometer (Thermo Electron Corporation). The controls without KSP or MTs were also measured to determine the background, which was subtracted from all measured values. The controls with MTs but without KSP give the nucleotide hydrolysis by MTs and should be subtracted from corresponding values with KSP and the same concentration of MTs. The data were analyzed using Microsoft Excel to obtain the IC_{50} of the test compounds. IC_{50} values are reported as the averages of at least three independent determinations; standard deviations are within ±25—50% of IC_{50} values.

***In Vitro* Cytotoxicity Assay (MTT Assay)** The tested cells were introduced into each well of a 96-well plate, with a density of 2500 cells/well. The cells were then exposed to compounds of different concentrations (1.0,

2.0, 4.0, 8.0, 16, 32 μM) (100 μl/well). Controls were performed in which only culture media was added into wells containing cells. After 48 h incubation, 5 mg/ml MTT solution (20 μl/well) was added and cultured for 4 h, and then the supernatant was discarded and DMSO was added in (100 μl/well), respectively. The suspension was placed on micro-vibrator for 5 min and the absorbance (A) was measured at 570 nm by the Universal Microplate Reader (EL800, BIO-TEK INSTRUMENTS INC.). Triplicate experiments were performed in a parallel manner for each concentration point and the results were reported presented as mean. Cell inhibitory ratio was calculated by the following formula:

$$\text{inhibitory ratio (\%)} = [(A_{\text{control}} - A_{\text{treated}})/A_{\text{control}}] \times 100\%$$

The IC_{50} was taken as the concentration that caused 50% inhibition of cell proliferation.

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