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Biological evaluation of KRIBB3 analogs as a microtubule polymerization inhibitor

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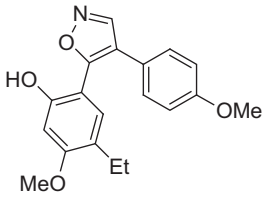
ABSTRACT

A series of KRIBB3 analogs were synthesized by modifying substituents at aryl moieties of KRIBB3 for examining structure–activity relationships, and their inhibitory activities on microtubule polymerization were evaluated. The presence of free phenolic hydrogens in aryl moieties of KRIBB3 analogs plays an important role in inhibition of microtubule polymerization.

Cancer is one of the major causes of death in human. Among various anticancer drug targets known to date, microtubules are one of the most successful targets for cancer therapy.[1] Microtubules are the principal components of the cytoskeletal system that takes part in intracellular transport, motility, architecture, and alignment and separation of chromosomes in meiosis and mitosis.[2] Microtubules are polymeric structures composed of two structurally similar protein subunits, namely α- and β-tubulin, arranged head-to-tail to form a linear protofilament. Small molecules that inhibit microtubules strongly block the proliferation of cancer cells. Traditional anti-microtubule drugs produce 'unattached' kinetochores in mitosis by altering microtubule dynamics and cause long-term mitotic arrest.[2,3] Vinca alkaloids which inhibit microtubule polymerization have been used in the treatment of cancer over 30 years.[4]

In previous studies, we reported a diaryl oxazole compound, KRIBB3 that displayed anti-mitotic activity against cancer cells (Fig. 1).[5] KRIBB3 exerted its antiproliferative activity through inhibition of microtubule polymerization.[5] In addition, unlike taxanes and vinblastine, KRIBB3 is not a substrate of P-glycoprotein (MDR1), which suggests that KRIBB3 is superior to other antimitotic agents in this regard. KRIBB3 showed 52% inhibition of microtubule polymerization compared with DMSO as a control. In an effort to further develop this promising compound toward a potent anticancer agent, we have focused on examining structure–activity relationships for KRIBB3. We synthesized a series of diaryl isoxazole derivatives by modifying substituents at aryl moieties of KRIBB3, and evaluated their inhibitory activity on microtubule polymerization. Herein, we describe the synthesis and biological evaluation of KRIBB3 analogs **4**.

The synthesis of diaryl isoxazole derivative **4b** was achieved through the route in Scheme 1. Alkylation of 4-ethylresorcinol (**1**) with benzyl bromide followed by addition of 4-methoxybenzylmagnesium chloride and the subsequent oxidation[6] of the resulting secondary alcohol with TPAP and NMO afforded ketone **2**. Enaminoketone **3** was prepared by condensation of compound **2** with dimethylformamide dimethylacetal (DMFDMA) in refluxing



KRIBB3

Figure 1.

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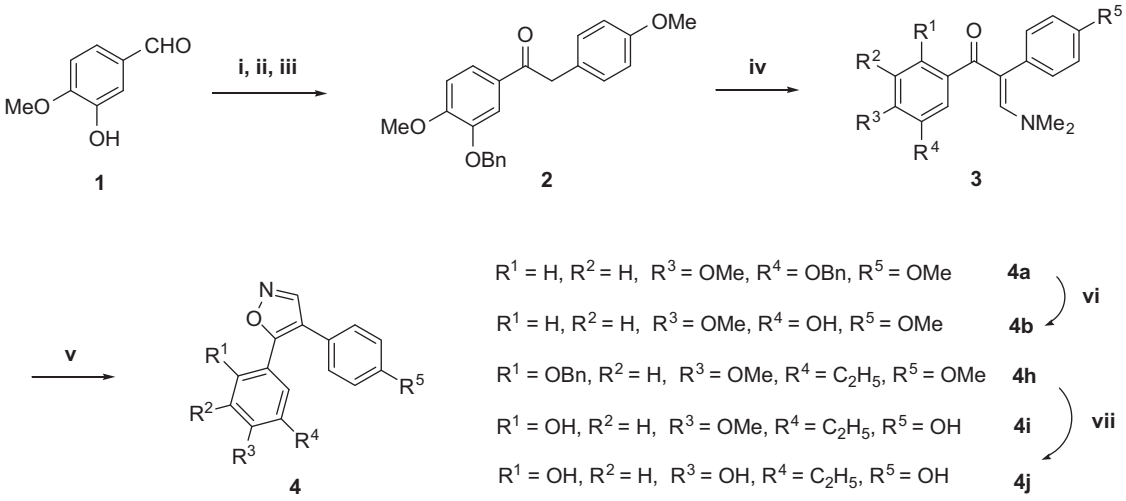
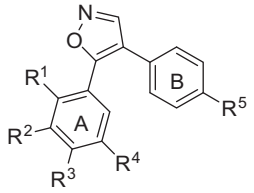


Scheme 1. Reagents and conditions: (i) $C_6H_5CH_2Br$, K_2CO_3, DMF, rt; (ii) 4-methoxybenzyl magnesium chloride, 0 °C to rt, 1 h; (iii) TPAP, 4-methylmorpholine *N*-oxide, 4 Å molecular sieves, CH_2Cl_2, rt, 30 min; (iv) DMFDMA, toluene, reflux; (v) NH_2OH–HCl, MeOH, AcOH, Na_2CO_3, 115 °C; (vi) H_2 (60 psi), 10% Pd/C, EtOAc; (vii) BBr_3, CH_2Cl_2, −10 °C, 2 h.

toluene.[7] Compound **3** was cyclized[7b] with hydroxylamine in refluxing methanolic AcOH in the presence of Na_2CO_3 to yield isoxazole **4a**.[8] Compound **4b** was synthesized by debenzylation of compound **4a** using hydrogen (60 psi) and 10% Pd/C.[9] Compounds **4c** (**4d**), **4e** (**4f**), **4g**, and **4h** were prepared from 3,4-dihydroxybenzaldehyde, 3,5-dimethyl-4-hydroxybenzaldehyde, 4-hydroxybenzaldehyde, and 2-benzyloxy-4-methoxy-5-ethylbenzaldehyde, respectively, in same manner as described for synthesis of **4a** and **4b**.[10] Compound **4i** and **4j** were prepared by demethylation of **4h** with treatment of BBr_3 at −10 °C for 2 h in 45% and 30% yields, respectively.

Inhibitory activity of diaryl isoxazole derivatives **4** against microtubule polymerization in comparison with KRIBB3 as a reference was listed in Table 1.[11] We found that the inhibition of microtubule polymerization was greater in methoxy moiety of B-aryl group of KRIBB3 analogs than in hydroxyl moiety (**4h** vs **4i** and **4j**). Then, we concentrated on modification of substituents at A-aryl moiety of KRIBB3 with the other aryl (B-aryl) substituent fixed as methoxy. The extent of inhibiting microtubule polymerization was lower in benzyloxy group of A-aryl moiety than in hydroxyl group (**4a** vs **4b**, **4c** vs **4d**, **4e** vs **4f**). The presence of phenolic hydrogen in the aryl moiety of KRIBB3 analogs **4** increased inhibitory activity against microtubule polymerization. The effect of inhibitory activity on the number of phenolic hydroxyl group was also examined. Compound **4d** with two phenolic hydrogens showed more potent than compound **4g** with one phenolic hydrogen. Interestingly, compound **4b** possessing one phenolic hydrogen and one methoxy group exhibited stronger inhibitory activity than compound **4d** possessing two phenolic hydrogens. However, compound **4f** with one phenolic hydrogen and two methyl groups showed less potent activity than compound **4d** with two phenolic hydrogens. Among the tested compounds, compound **4b** exhibited the strongest inhibitory activity against microtubule polymerization, more potent than KRIBB3 as a reference. Structure–activity analysis indicated that the existence of phenolic OH group in the A-aryl moiety of KRIBB3 analogs **4** was crucial to the inhibitory activity against microtubule polymerization, and compound **4b** possessing one phenolic OH group with one additional methoxy group showed stronger activity than those of two OH groups or one OH group.

Table 1
Inhibitory activity of diaryl isoxazole compounds against microtubule polymerization



Compound	R^1	R^2	R^3	R^4	R^5	Inhibition (%)
4a	H	H	OCH_3	$OCH_2C_6H_5$	OCH_3	128
4b	H	H	OCH_3	OH	OCH_3	50
4c	H	H	$OCH_2C_6H_5$	$OCH_2C_6H_5$	OCH_3	80
4d	H	H	OH	OH	OCH_3	65
4e	H	CH_3	$OCH_2C_6H_5$	CH_3	OCH_3	126
4f	H	CH_3	OH	CH_3	OCH_3	76
4g	H	H	OH	H	OCH_3	80
4h	$OCH_2C_6H_5$	H	OCH_3	C_2H_5	OCH_3	79
4i	OH	H	OCH_3	C_2H_5	OH	115
4j	OH	H	OH	C_2H_5	OH	134
KRIBB3[a]	OH	H	OCH_3	C_2H_5	OCH_3	52

[a] As a reference.

In summary, inhibitory activity was increased in methoxy moiety of B-aryl group of KRIBB3 analogs than in hydroxyl moiety. The effect of the number of phenolic hydrogens in A-aryl group of KRIBB3 analogs on inhibitory activities of microtubule polymerization was also examined for structure–activity relationships. KRIBB3 analogs lacking phenolic hydrogen showed poor inhibitory activity. The compound with two phenolic hydrogens in A-aryl group of KRIBB3 analogs **4** exhibited more potent than the compound with one phenolic hydrogen. Compound **4b** possessing one phenolic hydrogen and one methoxy group exhibited the strongest inhibitory activity.

Acknowledgments

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8. *Typical procedure for synthesis of compound* **4a**. To a mixture of 3-hydroxy-4-methoxybenzaldehyde (3.0 g, 19.7 mmol) and potassium carbonate (10.9 g, 78.8 mmol) in DMF (30 mL) was added benzyl bromide (4.7 mL, 39.4 mmol) and the reaction mixture was stirred at room temperature for 16 h. The reaction solution was filtered to remove inorganic salts, and the filtrate was diluted with water (200 mL), extracted three times with ethyl acetate and washed with brine. The combined organic layer was dried over anhydrous magnesium sulfate, filtered, and concentrated under reduced pressure. The concentrated residue was purified by silica-gel column chromatography (hexane/ethyl acetate = 5:1) to give 4.7 g of benzylated compound. To a mixture of magnesium turnings (0.75 g, 0.031 mol) in THF (5 mL) at room temperature was slowly added a solution of 4-methoxybenzylchloride (1.6 g, 10.2 mmol) in THF (10 mL). The reaction solution was refluxed with heating for one hour and then cooled down in a 0 °C water bath. The ashy solution was extracted by using a syringe, which was used as a Grignard reagent. To a solution of the benzylated aldehyde (0.82 g, 3.4 mmol) in THF (15 mL) at 0 °C was added the Grignard reagent slowly, and the reaction mixture was stirred at room temperature for 1 h. Saturated ammonium chloride solution was added to the reaction solution, and the mixture was extracted with ethyl acetate three times and washed with brine. Combined organic layer was dried over anhydrous magnesium sulfate, filtered, and concentrated under reduced pressure. The concentrated residue was purified by silica-gel column chromatography (hexane/ethyl acetate = 5:1), to give 1.2 g of alcoholic compound. To a mixture of the alcoholic compound (1.2 g, 3.3 mmol), 4-methylmorpholine *N*-oxide (0.57 g, 4.9 mmol) and anhydrous powdered 4 Å molecular sieves (1.64 g) in dichloromethane (10 mL) was added tetrapropylammonium perruthenate (57 mg). The reaction mixture was stirred for 30 min, passed through a short silica-gel pad by washing with ethyl acetate, and concentrated to give 1.1 g (88% in three-steps) of compound **2**. To a solution of compound **2** (1.26 g, 3.5 mmol) in toluene (10 mL) was added dimethylformamide dimethylacetal (DMFDMA) (1.2 g, 10 mmol). The reaction mixture was refluxed for 16 h at 135 °C. The reaction solution was cooled to 0 °C, concentrated and purified by silica-gel column chromatography (hexane/ethyl acetate = 1:2) to give 1.35 g (93%) of compound **3**. To a solution of compound **3** (1.12 g, 2.7 mmol) in methanol (35 mL) was added sodium carbonate (190 mg, 1.7 mmol) and NH_2OH HCl (1.9 g, 27.0 mmol). The mixture was adjusted to be pH 4–5 using acetic acid (1.0 mL) and then heated in a heavy-wall screw capped tube at 115 °C for 2 h. The reaction mixture was cooled to room temperature and methanol was removed under reduced pressure. The residue was extracted with methylene chloride and water, and the organic layer washed with brine, dried over Na_2SO_4, concentrated and purified by silica-gel column chromatography (hexane/ethyl acetate = 5:1) to give 1.0 g (97%) of isoxazole compound **4a**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.33–7.27 (m, 7H), 7.25 (dd, *J* = 7.2, 2.4 Hz, 1H), 7.20 (d, *J* = 1.8 Hz, 1H), 6.93 (dd, *J* = 6.9, 2.7 Hz, 2H), 6.87 (d, *J* = 8.7 Hz, 1H), 5.00 (s, 2H), 3.90 (s, 3H), 3.85 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.4, 159.4, 151.9, 151.0, 148.1, 136.6, 130.0, 128.5, 127.9, 127.2, 122.5, 120.7, 120.4, 114.7, 114.4, 112.5, 111.6, 70.9, 55.9, 55.3; HRMS (FAB) *m*/*z* 340.1558 [(M+H)$^+$, calcd for $C_{24}H_{22}NO_4$ 388.1549].
9. *Compound* **4b**. To a solution of the isoxazole compound **4a** (1.0 g, 2.58 mmol) in ethyl acetate (10 mL) was added 10% palladium/c (40 mg). The reaction was performed under 60 psi of hydrogen atmosphere for 14 h. The reaction solution was passed through a short silica-gel pad by washing with ethyl acetate, concentrated, and purified by silica-gel column chromatography (hexane/ethyl acetate = 1:2) to give 0.7 g (90%) of **4b**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.27 (s, 1H), 7.30 (dd, *J* = 6.3, 2.1 Hz, 2H), 7.21 (d, *J* = 1.8 Hz, 1H), 7.17 (dd, *J* = 8.7, 2.4 Hz, 1H), 6.92 (dd, *J* = 6.3, 2.1 Hz, 2H), 6.84 (d, *J* = 8.4 Hz, 1H), 5.67 (s, 1H), 3.92 (s, 3H), 3.84 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.4, 159.4, 152.0, 147.9, 145.6, 129.9, 122.4, 121.1, 119.7, 114.9, 114.4, 113.3, 110.6, 55.9, 55.2; HRMS (FAB) *m*/*z* 297.1068 [(M+H)$^+$, calcd for $C_{17}H_{16}NO_4$ 298.1079].
10. *Compound* **4c**. Compound **4c** was prepared from 3,4-dihydroxybenzaldehyde according to the typical procedure: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.26 (s, 1H), 7.45–7.26 (m, 13H), 7.20 (dd, *J* = 8.4, 2.4 Hz, 1H), 6.94 (dd, *J* = 6.9, 2.4 Hz, 2H), 6.91 (d, *J* = 7.8 Hz, 1H), 5.19 (s, 2H), 5.04 (s, 2H), 3.86 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.3, 159.4, 152.0, 150.2, 148.8, 136.7, 130.0, 128.5, 128.4, 127.9, 127.8, 127.2, 127.1, 122.5, 121.0, 120.7, 114.8, 114.4, 113.4, 71.1, 71.0, 55.3; HRMS (FAB) *m*/*z* 464.1836 [(M+H)$^+$, calcd for $C_{30}H_{26}NO_4$ 464.1862].
Compound **4d**. Compound **4d** was prepared from compound **4c** by hydrogenation as described for synthesis of **4b**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.40 (br s, 2H), 8.71 (s, 1H), 7.33 (dd, *J* = 6.9, 2.1 Hz, 2H), 6.98 (dd, *J* = 6.9, 2.1 Hz, 2H), 6.97 (s, 1H), 6.88 (dd, *J* = 7.8, 1.8 Hz, 1H), 6.78 (d, *J* = 8.4 Hz, 1H), 3.78 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 163.1, 158.7, 152.1, 147.4, 145.4, 129.5, 121.9, 118.7, 115.9, 114.3, 114.0, 113.8, 55.0; HRMS (FAB) *m*/*z* 284.0917 [(M+H)$^+$, calcd for $C_{16}H_{14}NO_4$ 284.0923].
Compound **4e**. Compound **4e** was prepared from 3,5-dimethyl-4-hydroxybenzaldehyde according to the typical procedure: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.30 (s, 1H), 7.49–7.30 (m, 9H), 6.95 (m, 2H), 4.84 (s, 2H), 3.85 (s, 3H), 2.26 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.6, 159.3, 157.2, 151.8, 137.3, 131.7, 129.7, 128.5, 128.1, 127.8, 127.7, 123.5, 122.5, 115.1, 114.3, 70.0, 63.8, 55.2, 16.4; HRMS (FAB) *m*/*z* 386.1748 [(M+H)+, calcd for $C_{25}H_{24}NO_3$ 386.1756].
Compound **4f**. Compound **4f** was prepared from compound **4e** by hydrogenation as described for synthesis of compound **4b**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.28 (s, 1H), 7.31 (dd, *J* = 6.6, 1.8 Hz, 2H), 7.28 (br s, 2H), 6.92 (dd, *J* = 6.6, 2.4 Hz, 2H), 4.91 (s, 1H), 3.85 (s, 3H), 2.21 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.9, 159.2, 153.7, 151.8, 129.7, 127.7, 123.3, 122.7, 119.9, 114.4, 114.3, 53.3, 15.8; HRMS (FAB) *m*/*z* 296.1284 [(M+H)$^+$, calcd for $C_{18}H_{18}NO_3$ 296.1287].
Compound **4g**. Compound **4g** was prepared from 4-hydroxybenzaldehyde according to the typical procedure and the subsequent hydrogenation: ^{1}H NMR (300 MHz, CD_3OD) δ 8.41 (s, 1H), 7.42 (dd, *J* = 6.6, 1.8 Hz, 2H), 7.29 (dd, *J* = 6.6, 1.8 Hz, 2H), 6.94 (dd, *J* = 6.6, 2.4 Hz, 2H), 6.78 (dd, *J* = 6.6, 2.4 Hz, 2H), 4.85 (s, 1H), 3.80 (s, 3H); ^{13}C NMR (75 MHz, CD_3OD) δ 165.3, 160.9, 160.5, 153.0, 130.9, 129.8, 123.7, 120.2, 116.6, 115.8, 115.4, 55.7; HRMS (FAB) *m*/*z* 268.0968 [(M+H)$^+$, calcd for $C_{16}H_{14}NO_3$ 268.0974].
Compound **4h**. Compound **4h** was prepared from 2-benzyloxy-4-methoxy-5-ethylbenzaldehyde according to the typical procedure: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.42 (s, 1H), 7.25–7.00 (m, 8H), 6.78 (dd, *J* = 6.9, 2.4 Hz, 2H), 6.48 (s, 1H), 4.84 (s, 2H), 3.80 (s, 3H), 3.78 (s, 3H), 2.56 (q, *J* = 7.8 Hz, 2H), 1.14 (t, *J* = 7.8 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 162.3, 159.7, 158.7, 155.5, 150.5, 136.5, 130.8, 128.2, 127.6, 127.0, 125.3, 123.3, 116.6, 113.8, 109.3, 97.0, 70.7, 55.3, 55.2, 22.3, 14.0; HRMS (FAB) *m*/*z* 416.1859 [(M+H)$^+$, calcd for $C_{26}H_{26}NO_4$ 416.1862].
11. *Microtubule polymerization assay*. For the detection of polymerization of tubulin/microtubule, CytoDYNAMIX Screen 01 kits were purchased form Cytoskeleton Inc. (Denver, CO) and polymerization assay was done as described previously.[4] Tubulin proteins (>97% purity) were suspended (300 μg/sample) with 100 μl of G-PEM buffer (80 mM PIPES, 2 mg $MgCl_2$, 0.5 mM EGTA, 1.0 mM GTP, pH 6.9) plus 5% glycerol in the 0.1% DMSO or test compounds at 4 °C. Then the sample mixture was transferred to the pre-warmed 96-well plate, and polymerization of tubulin was measured by the change in absorbance at 340 nm every 1 min for 70 min (Wallac victor2; PerkinElmer, Inc., Wellesley, MA) at 37 °C. Inhibitory activity of KRIBB3 or its analogs was calculated using initial polymerization activity (from 0 to 10 min). To compare each compound's inhibitory activity, we used initial polymerization reaction slop (from 0 to 10 min). Slop of polymerization reaction was calculated using linear equation method with Sigma Plot Program and slop of DMSO treated reaction was used as a control. Relative inhibition of tube formation is percentage of slop for KRIBB3 analogs compared to the slop for DMSO treated reaction.