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Bioorganic & Medicinal Chemistry 14 (2006) 528–536
Bioorganic & Medicinal Chemistry

Synthesis of N^2-(substituted benzyl)-3-(4-methylphenyl)indazoles as novel anti-angiogenic agents

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Received 10 June 2005; revised 16 August 2005; accepted 17 August 2005
Available online 3 October 2005

Abstract—To search for novel compounds with potent anti-angiogenic activity, a series of N^1-(substituted benzyl)-3-(4-methylphenyl)-1*H*-indazoles (**16**, **18**, **20**, **22**, **24**, **26**, **28**, **30**, **32**) and N^2-(substituted benzyl)-3-(4-methylphenyl)-2*H*-indazoles (**17**, **19**, **21**, **23**, **25**, **27**, **29**, **31**, and **33**) were synthesized. The structures of these regioisomers were established by IR, UV, and NMR spectral data. 3-(4-Methylphenyl)-1*H*-indazole (**6**) and the N^2-substituted derivatives (**17**, **19**, **21**, **23**, **25**, **29**, **31**, **33**) were evaluated for their anti-angiogenic activity. Most of them showed more prominent activity than ethyl 4-(1-benzyl-1*H*-indazol-3-yl)benzoate (**YD-3**). Among these tested compounds, 2-(4-chlorobenzyl)-3-(4-methylphenyl)-2*H*-indazole (**19**), 2-(4-methylbenzyl)-3-(4-methylphenyl)-2*H*-indazole (**25**), and 2-(4-methoxybenzyl)-3-(4-methylphenyl)-2*H*-indazole (**31**) showed significant anti-angiogenic activity and are worthy of further investigation.

1. Introduction

Angiogenesis, the formation of new capillaries from pre-existing blood vessel, is essential in physiologic and pathologic processes.[1] The first hypothesis that angiogenesis is essential for tumor growth and metastasis was made by Folkman about 30 years ago.[2] Since then, the research activity in the field of angiogenesis has increased immensely. A number of angiogenic agents are currently under advanced stage clinical trials for the treatment of cancer. Among them, bevacizumab (Avastin),[3] in combination with chemotherapy, was approved by the FDA in 2004.

Recently, a series of N^1-(substituted benzyl)-3-(substituted aryl)indazoles were evaluated for their anti-angiogenic activity. Among these tested compounds, ethyl 4-(1-benzyl-1*H*-indazol-3-yl)benzoate (**YD-3**) exhibited significant anti-angiogenic activity.[4]

In this work, the previously unknown N^2-regioisomers of indazole derivatives were synthesized and their anti-angiogenic activities were examined.

2. Results and discussion

2.1. Chemistry

The synthesis of N^1- and N^2-(substituted benzyl)-3-(4-methylphenyl)indazoles (**16**–**33**) is illustrated in Scheme 1. In the beginning, 1-(*N*-morpholino)cyclohexene (**1**) was treated with 4-methylbenzoyl chloride (**2**) in the presence of Et_3N to produce 4-[2-(4-methylbenzoyl)cyclohexylidene]morpholin-4-ium (**3**) which was then acidified with 20% HCl and heated to yield 2-oxocyclohexyl-4-methyl phenyl ketone (**4**).[5] Next, the condensation of compound **4** with hydrazine hydrate afforded 3-(4-methylphenyl)-4,5,6,7-tetrahydro-1*H*-indazole (**5**).[6] Subsequent dehydrogenation of compound **5** over Pd/C, under elevated temperature, yielded the key intermediate, 3-(4-methylphenyl)-1*H*-indazole (**6**).[7] Afterwards, compound **6** was subjected to alkylation by treating with various substituted benzyl chlorides in the presence of EtONa, to yield the corresponding N^1- and N^2-regioisomers (**16**–**33**). To avoid the

Keywords: Anti-angiogenic agents; N^2-(Substituted benzyl)-3-(4-methylphenyl)-indazoles.
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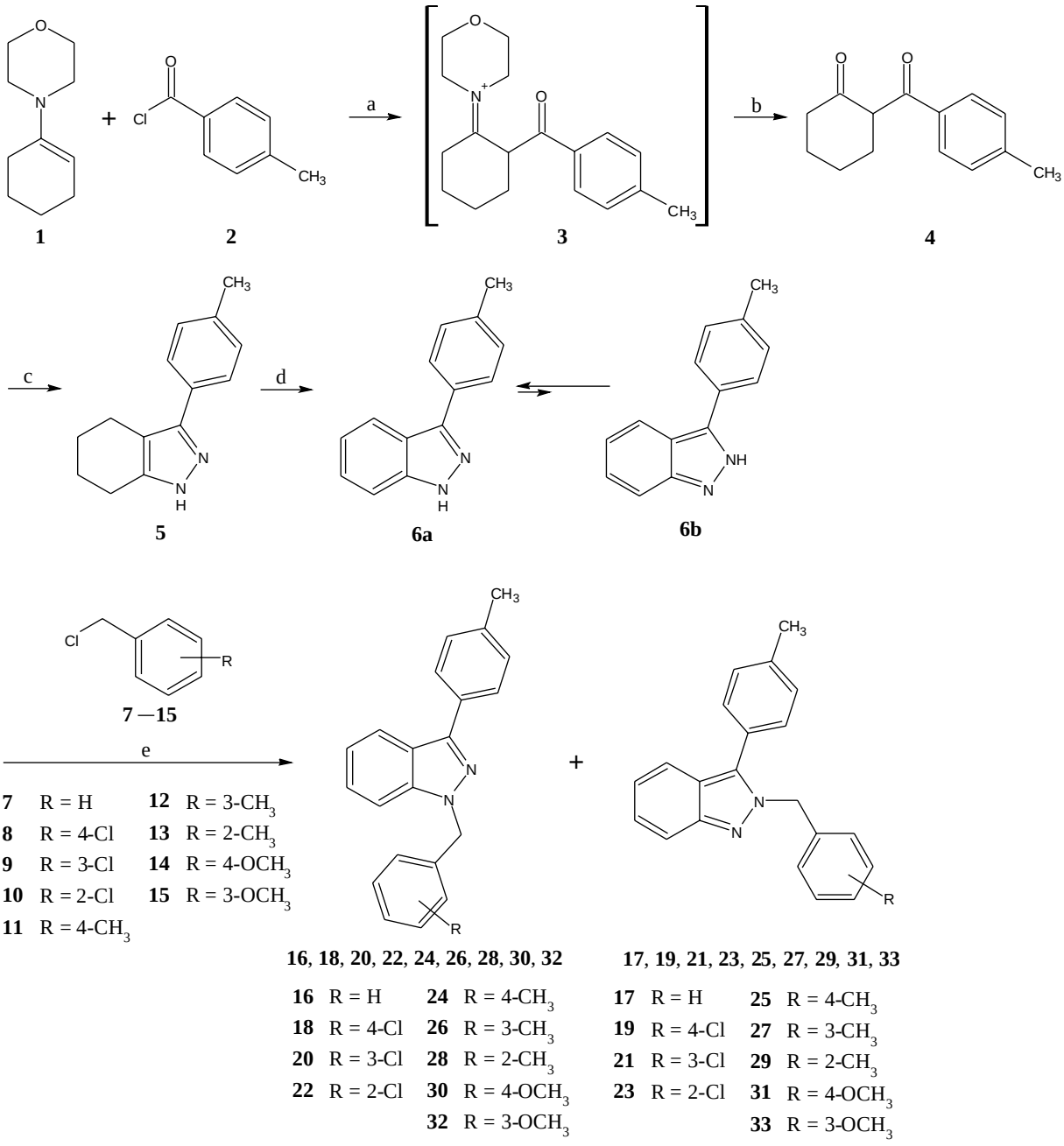
doi:10.1016/j.bmc.2005.08.032

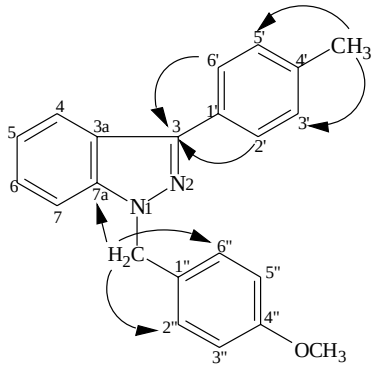


Scheme 1. Reagents and conditions: (a) Et_3N, $CHCl_3$, 45 °C, 3 h; (b) 20% HCl, reflux, 5 h; (c) 85% $NH_2NH_2{\cdot}H_2O$, MeOH, 30 °C, 30 min; (d) *trans*-decalin, 10% Pd/C, reflux, 24 h; (e) substituted benzyl chloride, EtONa, EtOH, reflux, 1.5 h.

redundancy in describing the structural verification, discussion for all resulted regioisomers, only the details of structural determination for compounds **30** (mp 67–69 °C) and **31** (mp 113–115 °C) were provided in the following as representative examples.

For both compounds **30** and **31**, elemental analysis and mass spectral data [*m*/*z* 328 (M^+)] established their molecular formula as $C_{22}H_{20}NO$, suggesting that they are possibly the N^1- and N^2-*p*-methoxybenzyl regioisomers. Although IR, UV, MS, and 1D-NMR spectral analysis could not distinguish them, their HMBC spectra were used successfully to confirm their isomeric structures. As shown in Figure 1, the signal of the *N*-CH_2-moiety of compound **30** showed ^3J-correlation with its 7a-, 2″-, and 6″-carbons. In contrast, the signal of the *N*-CH_2-moiety of compound **31** exhibited ^3J-correlation with its 3-, 2″-, and 6″-carbons. Based on the above data, compound **30** was identified as N^1-(*p*-methoxybenzyl)-3-(4-methylphenyl)indazole and compound **31** was confirmed as N^2-(*p*-methoxybenzyl)-3-(4-methylphenyl)indazole. The structures for the rest of the *N*-(substituted benzyl) derivatives (**16**–**29**, **32**, and **33**) could be assigned by similar spectral analysis procedures.

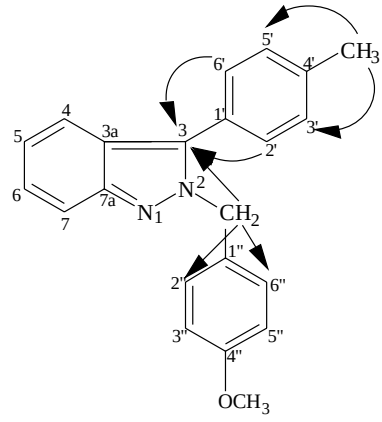
After comparing the physical and spectral data of these regioisomers, it was discovered that the melting points of all the N^1-regioisomers were relatively lower than



Compound 30

HMBC correlations	
^{1}H	**^{13}C**
H-4	C-6, C-7a
H-5	C-3a, C-7
H-6	C-4, C-7a
H-7	C-3a, C-5
H-2″, 6″	-$\underline{C}H_2$-, C-6″, 2″, C-4″
H-3″, 5″	C-1″, C-5″, 3″, C-4″*
H-2′, 6	C-3, C-4′, C-6′, 2′
H-3′, 5′	4′-$\underline{C}H_3$, C-1′, C-5′, 3′
4″-OC$\underline{H}_3$	C-4″
4′-C$\underline{H}_3$	C-5′, 3′, C-4′*
N-C$\underline{H}_2$	C-2″, 6″, C-7a

*: $^{2}J_{CH}$ correlation; others are $^{3}J_{CH}$ correlation



Compound 31

HMBC correlations	
^{1}H	**^{13}C**
H-4	C-6, C-7a
H-5	C-3a, C-7
H-6	C-4, C-7a
H-7	C-3a, C-5
H-2″, 6″	-$\underline{C}H_2$-, C-6″, 2″, C-4″
H-3″, 5″	C-1″, C-5″, 3″, C-4″*
H-2′, 6′	C-3, C-4′, C-6′, 2′
H-3′, 5′	4′-$\underline{C}H_3$, C-1′, C-5′, 3′
4″-OC$\underline{H}_3$	C-4″
4′-C$\underline{H}_3$	C-5′, 3′, C-4′*
N-C$\underline{H}_2$	C-2″, 6″, C-3

*: $^{2}J_{CH}$ correlation; others are $^{3}J_{CH}$ correlation

Figure 1. HMBC correlations of compounds **30** and **31**.

those of their corresponding N^2-regioisomers, and that the similar spectral patterns were observed for the same type of regioisomers. For instance, in the ^{1}H NMR spectra of all the N^1-regioisomers (**16**, **18**, **20**, **22**, **24**, **26**, **28**, **30**, and **32**), the signals of four protons appeared in low field correlated well with their H-4, H-2′, H-6′, and H-7, respectively, in the same order of decreasing chemical shift. Similarly, in all their ^{13}C NMR spectra, the signals of their quaternary carbons also correlated nicely with their C-3, C-7a, C-4′, C-1′, and C-3a, respectively, in the same order of decreasing chemical shift. In contrast, it was discovered that both the ^{1}H NMR and ^{13}C NMR spectra of the N^2-regioisomers (**17**, **19**, **21**, **23**, **25**, **27**, **29**, **31**, and **33**) differed slightly from those of their corresponding N^1-regioisomers. In the N^2-regioisomers, the signals of their four low field protons were assigned to H-7, H-4, H-2′, and H-6′, respectively, in the order of decreasing chemical shift. Meanwhile, the ^{13}C NMR signals of their five quaternary carbons were correlated with C-7a, C-4′, C-3, C-1′, and C-3a, respectively, in the order of decreasing chemical shift.

It is worthwhile mentioning that our observations of the difference in mp and NMR spectra between the above regioisomers can be used as valuable reference when dealing with structure verification of related regioisomers.

Our attempt to prepare the N^2-regioisomer of **YD-3**, by treating compound **6** or **16** with oxidizing agents like CrO_3, $KMnO_4$, or NBS,[8–10] however, did not afford the desired products, but resulted in a mixture of products that were not easily separable. Thus, we are still pursuing an alternative synthetic method and will report the outcome separately.

2.2. Anti-angiogenic activity

In this study, five of the above-mentioned N^2-regioisomers, namely, compounds **17**, **19**, **21**, **25**, and **31** were selected for evaluating their effects on VEGF-induced cell proliferation, and on neovascular formation in vivo. The results are reported in the following.

2.3. Effect on VEGF-induced cell proliferation of HUVECs

The effect of tested compounds on VEGF-induced cell proliferation of HUVECs was assessed with [^{3}H]thymidine incorporation assay, and the results are illustrated in Figures 2–4. At concentrations of 10 and 30 μM (Figs. 2 and 3), all of the six tested compounds (**6**, **17**, **19**, **21**, **25**, and **31**) exhibited significant inhibitory effect. When their concentration was lowered to 1 μM (Fig. 4), only compounds **19**, **25**, and **31** maintained better activity than positive control **YD-3**.

2.4. Cytotoxicity of compounds 6, 17, 19, 21, 25, and 31

The cytotoxicity of the six tested compounds (**6**, **17**, **19**, **21**, **25**, and **31**) is illustrated in Table 1; the IC_{50} values of these compounds against HL-60 cell lines were all found to be greater than 36 μM, indicating that they are not non-specific cytotoxic agents.

2.5. Effect on neovascular formation in vivo

The in vivo Matrigel plug assay was used in this study to determine quantitatively the anti-angiogenic effect of tested compounds. As shown in Figures 5 and 6, all of the tested N^2-regioisomers (**17**, **19**, **21**, **25**, and **31**) demonstrated significant anti-angiogenic activity. Among them, compounds **19**, **25**, and **31** showed superior potency to the positive control **YD-3**.

Analysis of the anti-angiogenic activity of our tested compounds led to the important finding that the introduction of Cl, CH_3, or OCH_3 group into the *para*-position of the N^2-benzyl group of the tested indazole derivatives is significantly beneficial for their anti-angiogenic activity.

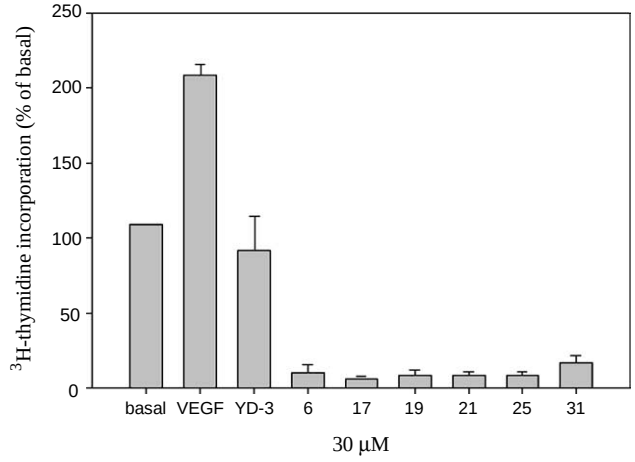


Figure 2. In vitro assay (inhibition of DNA synthesis). Human umbilical vein endothelial cells were incubated in the absence (basal and control) or presence of tested sample (**YD-3**, **6**, **17**, **19**, **21**, **25**, and **31**), and then vascular endothelial growth factor (VEGF) was added (except for basal) to induce DNA synthesis, which was detected using [^{3}H]thymidine incorporation assay. Means ± SE (n = 5) are presented.

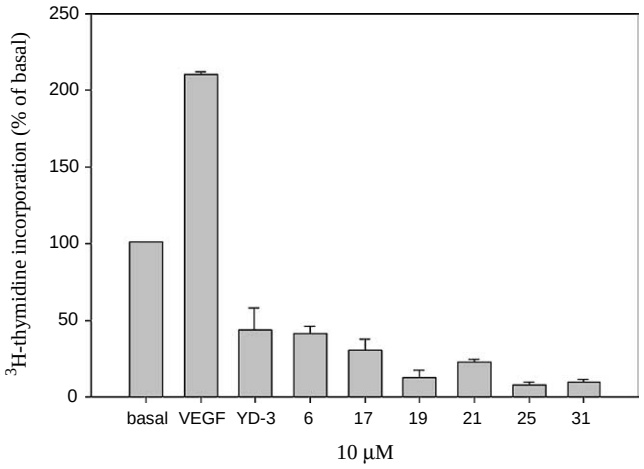


Figure 3. In vitro assay (inhibition of DNA synthesis). Human umbilical vein endothelial cells were incubated in the absence (basal and control) or presence of tested sample (**YD-3**, **6**, **17**, **19**, **21**, **25**, and **31**), and then vascular endothelial growth factor (VEGF) was added (except for basal) to induce DNA synthesis, which was detected using [^{3}H]thymidine incorporation assay. Means ± SE (n = 5) are presented.

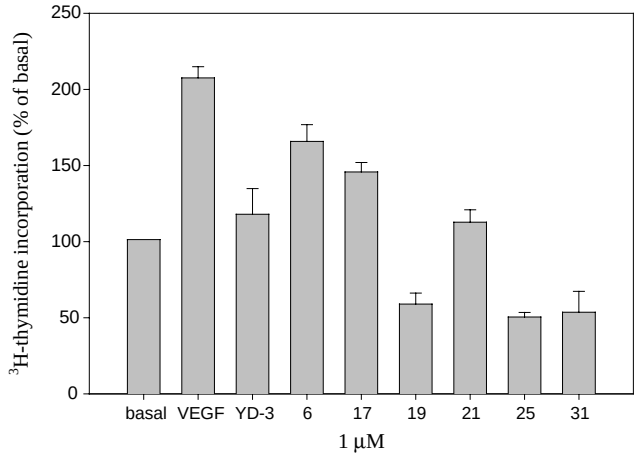
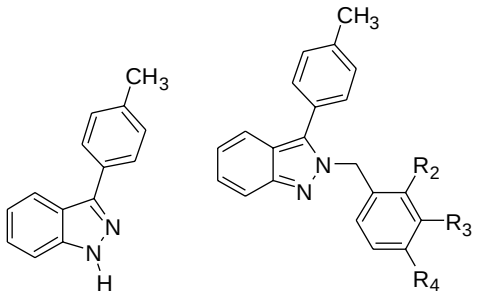


Figure 4. In vitro assay (inhibition of DNA synthesis). Human umbilical vein endothelial cells were incubated in the absence (basal and control) or presence of tested sample (**YD-3**, **6**, **17**, **19**, **21**, **25**, and **31**), and then vascular endothelial growth factor (VEGF) was added (except for basal) to induce DNA synthesis, which was detected using [^{3}H]thymidine incorporation assay. Means ± SE (n = 5) are presented.

Based on their anti-angiogenic activity, we have identified that, within the series of N^2-substituted phenyl-3-(4-methylphenyl)indazole derivatives, compounds **19**, **25**, and **31** exhibited superior activity to other derivatives and will be targeted for further exploration.

However, some of the N^1-derivatives are known compounds with structure and anti-angiogenic activity relationship very different from N^2-derivatives. Such biological data will be published separately.

Table 1. Cytotoxic effect of compounds **6**, **17**, **19**, **21**, **25**, **31**



6 **17, 19, 21, 25, 31**

No.	R_4	R_3	R_2	Concn (μM)	MTT assays (%)
Control				0	100 ± 0.1
6	—	—	—	50	79.5 ± 6.9 **
				25	102.7 ± 5.2
				10	96.0 ± 10.5
IC_{50} > 50 μM					
17	H	H	H	50	78.0 ± 2.3***
				25	103.0 ± 4.4
				10	108.2 ± 0.2***
IC_{50} > 50 μM					
19	Cl	H	H	50	32.9 ± 7.2
				25	57.9 ± 4.8***
				10	92.6 ± 7.2
IC_{50} = 36.1 μM					
21	H	Cl	H	50	44.1 ± 4.5***
				25	72.4 ± 3.3***
				10	92.7 ± 3.4**
IC_{50} = 44.7 μM					
25	CH_3	H	H	50	40.8 ± 6.1***
				25	71.9 ± 2.0***
				10	94.3 ± 3.0 *
IC_{50} = 42.6 μM					
31	OCH_3	H	H	50	33.1 ± 2.0***
				25	61.4 ± 3.1***
				10	87.4 ± 4.1**
IC_{50} = 36.3 μM					

HL-60 cells (1×10^5/mL) were treated with tested sample for 24 h. Data are presented as means ± SD from three separate experiments.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$, compared with control.

3. Experimental

All of the solvents and reagents were obtained commercially and used without further purification. Reactions were monitored by thin-layer chromatography, using Merck plates with fluorescent indicator. Column chromatography was performed on silica gel.

Melting points were determined with a Yanaco MP-500D melting point apparatus and are uncorrected. IR spectra were recorded on Shimadzu IR-440 and Nicolet Impact 400 FT-IR spectrophotometers as KBr pellets. NMR spectra were obtained on a Bruker Avance DPX-200 FT-NMR spectrometer in $CDCl_3$. The following abbreviations are used: s, singlet; d, doublet; dd, double doublet; m, multiplet; and br, broad. MS spectra were measured with an HP 5995 GC–MS instrument. The UV spectra were recorded on a Shimadzu UV–160A UV–vis recording spectrophotometer as methanolic solutions. Elemental analyses (C, H, and N) were performed on a Perkin-Elmer 2400 Series II CHNS/O analyzer and the results were within ±0.4% of the calculated values.

3.1. Preparation of *N*-(substituted benzyl)-3-(4-methylphenyl)indazoles (16–33)

3.1.1. 3-(4-Methylphenyl)-1*H*-indazole (6). (A) Into a solution of 1-(*N*-morpholino)cyclohexene (**1**) (33.4 g, 0.2 mol) and triethylamine (28 mL) in $CHCl_3$ (100 mL) was added dropwise the solution of 4-methylbenzoyl chloride (**2**) (30.8 g, 0.2 mol) in $CHCl_3$ (40 mL) at 45 °C. The reaction mixture was allowed to react for 3 h. Then 20% HCl was added, and the mixture was heated under reflux for 5 h and then allowed to stand at room temperature. The $CHCl_3$ layer was collected and washed with H_2O dried over $MgSO_4$, and then evaporated. The residue was washed with petroleum ether and dried to yield 2-oxycyclohexyl-4-methylphenyl ketone (**4**), yield 21.2 g, 49%; mp 106–107 °C.

(B) Compound **4** (13.01 g, 0.06 mol) was dissolved in MeOH (100 mL) and then 4 mL of 85% $NH_2NH_2{\cdot}H_2O$ was added dropwise at 30 ± 2 °C. The mixture was allowed to react for 30 min and was then concentrated in vacuo until about 35 mL solution was left. The residue was allowed to precipitate during cooling. The solid precipitate was washed with petroleum ether and dried to yield 3-(4-methylphenyl)-4,5,6,7-tetrahydro-1*H*-indazole (**5**), yield 12.39 g, 97%; mp 57–60 °C.

(C) Into the solution of compound **5** (12.3 g, 0.058 mol) in *trans*-decahydronaphthalene (*trans*-decalin) was added 10% Pd/C (2.7 g), and the mixture was heated under reflux for 24 h, and then concentrated in vacuo in oil bath until about 20 mL solution was left. Petroleum ether (80 mL) was added into the residue while hot, and the mixture was well mixed by shaking. The mixture was allowed to precipitate upon cooling to afford 3-(4-methylphenyl)-1*H*-indazole (**6**), yield 9.85 g, 82%; mp 63–65 °C; MS (EI, 70 eV): *m*/*z* 208 (M^+); found: C, 80.70; H, 5.75; N, 13.40. $C_{14}H_{12}N_2$ requires: C, 80.74; H, 5.81; N, 13.45; UV λ_{max} ($\log\varepsilon$): 233.4 (3.85), 246.0 (4.22), 311.6 (4.25); IR (KBr): 1479 (C=N) cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): δ 2.47 (s, 3H), 7.19–7.29 (m, 2H), 7.34–7.38 (m, 3H), 7.93 (d, 2H, J = 8.0 Hz), 8.03 (d, 1H, J = 8.1 Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ 21.33, 110.26, 120.92, 121.17, 126.70, 127.60, 129.62, 130.65, 138.02, 141.66, 145.68.

3.1.2. 1-Benzyl-3-(4-methylphenyl)-1*H*-indazole (16) and 2-benzyl-3-(4-methylphenyl)-2*H*-indazole (17). Into the solution of compound **6** (6.24 g, 0.03 mol) in anhydrous

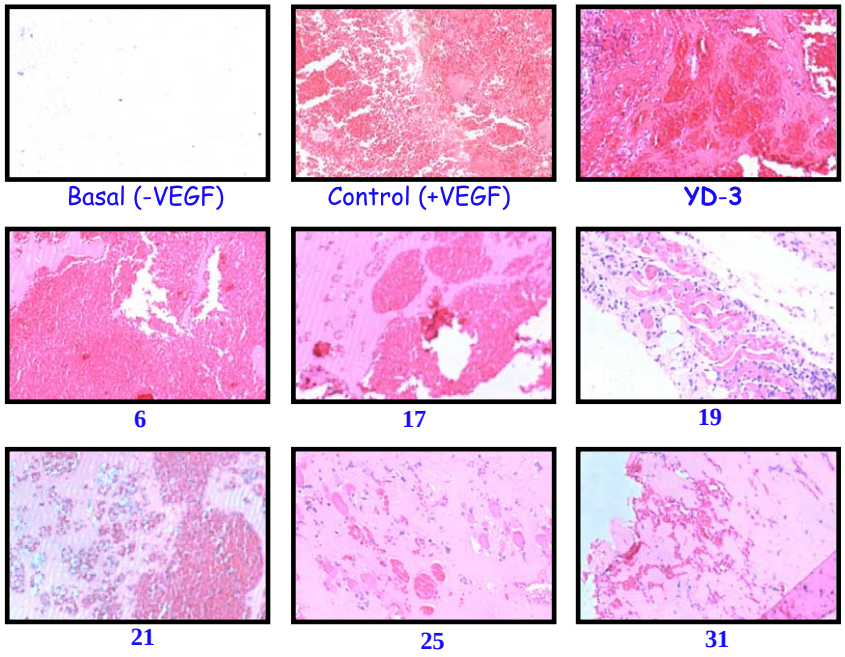


Figure 5. In vivo mouse Matrigel-plug assay. Nude mice were subcutaneously injected with a Matrigel plug containing 150 ng/mL vascular endothelial growth factor (VEGF). Vehicle or 10 μm tested sample (**YD-3**, **6**, **17**, **19**, **21**, **25**, and **31**) was coadministered into the mice. After a seven-day administration, the animals were euthanatized and the plugs were cut off the mice for the measurement of angiogenic effect using histological analysis (H&E staining). Means ± SE ($n = 3$) are presented.

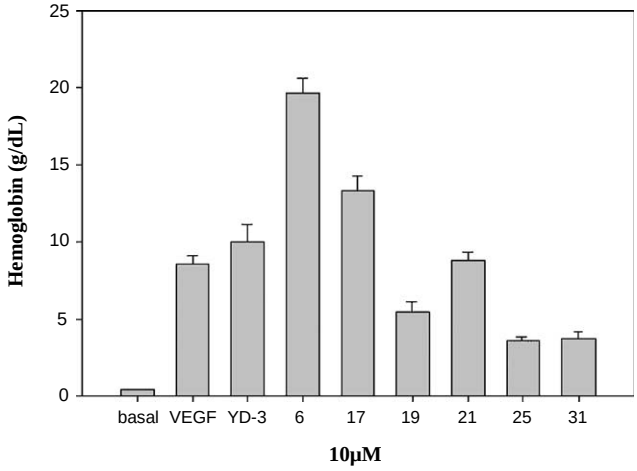


Figure 6. Quantitative analysis of angiogenic effect. Nude mice were subcutaneously injected with a Matrigel plug containing 150 ng/mL vascular endothelial growth factor (VEGF). Vehicle or 10 μm tested sample (**YD-3**, **6**, **17**, **19**, **21**, **25**, and **31**) was coadministered into the mice. After a seven-day administration, the animals were euthanatized and the plugs were cut off the mice for the measurement of angiogenic effect using the hemoglobin concentration as the parameter by means of a hemoglobin detection kit (Sigma). Means ± SE ($n = 3$) are presented.

EtOH (30 mL) was added EtONa (4.08 g, 0.06 mol). The mixture was stirred at 30 ± 2 °C for 1.5 h. Benzyl chloride (**7**) (10.08 g, 0.08 mol) was added dropwise, and mixture was heated under reflux for 1.5 h. The solid precipitate so formed was filtered off while hot and washed several times with $CHCl_3$. The combined filtrate was concentrated in vacuo for solvent removal. The residue was chromatographed (silica gel–$CHCl_3$) to produce compounds **16** and **17**. Compound **16**: yield 3.6 g, 40%; mp 41–42 °C; MS (EI, 70 eV): *m/z* 298 (M^+); found: C, 84.48; H, 6.09; N, 8.99. $C_{20}H_{18}N_2$ requires: C, 80.46; H, 6.14; N, 8.53; UV λ_{max} ($\log\varepsilon$): 233.8 (3.63), 242 (3.96), 313.6 (3.95); IR (KBr): 1509 (C═N) cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): δ 2.35 (s, 3H), 5.71 (s, 2H), 7.17–7.37 (m, 8H), 7.40–7.45 (m, 1H), 7.74 (d, 1H, $J = 8.2$ Hz), 7.88 (d, 2H, $J = 8.1$ Hz), 8.05 (d, 1H, $J = 8.2$ Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ 21.09, 52.08, 110.47, 121.18, 121.25, 121.47, 127.58, 129.71, 130.68, 137.43, 137.70, 141.10, 143.02. Compound **17**: yield 0.45 g, 5%; mp 110–111 °C; MS (EI, 70 eV): *m/z* 298 (M^+): found: C, 84.50; H, 6.02; N, 9.34. $C_{20}H_{18}N_2$ requires: C, 80.46; H, 6.14; N, 8.53; UV λ_{max} ($\log\varepsilon$): 233.2 (3.64), 243 (3.95), 314.6 (3.97); IR (KBr): 1497 (C═N) cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): δ 2.37 (s, 3H), 5.50 (s, 2H), 6.92–7.23 (m, 10H), 7.48 (d, 1H, $J = 8.4$ Hz), 7.66 (d, 1H, $J = 8.7$ Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ 21.36, 53.56, 117.31, 120.42, 121.27, 121.86, 126.43, 126.53, 128.32, 128.82, 129.49, 129.75, 133.56, 135.41, 136.67, 139.07, 148.34.

3.1.3. 1-(4-Chlorobenzyl)-3-(4-methylphenyl)-1*H*-indazole (18) and 2-(4-chlorobenzyl)-3-(4-methylphenyl)-2*H*-indazole (19). Compound **6** (4.16 g, 0.02 mol), EtONa (6.8 g, 0.1 mol), and 4-chlorobenzyl chloride (**8**) (16.1 g, 0.1 mol) were allowed to react as in the preparation of compound **16** and compound **17** to afford compound **18** and compound **19**. Compound **18**: yield 3.1 g, 47%; mp 80–82 °C; MS (EI, 70 eV): *m/z* 332 (M^+); found: C, 75.74; H, 5.13; N, 8.41. $C_{21}H_{17}N_2$ requires: C, 75.78; H, 5.15; N, 8.42; UV λ_{max} ($\log\varepsilon$): 232.2 (3.76), 244.6 (4.07), 313.4 (4.09); IR (KBr): 1492 (C═N) cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): δ 2.35 (s,

3H), 5.52 (s, 2H), 7.06–7.28 (m, 9H), 7.79 (d, 2H, $J = 8.1$ Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ 21.33, 52.26, 109.34, 121.08, 121.59, 122.13, 126.48, 127.38, 128.48, 128.85, 129.52, 130.61, 133.52, 135.42, 137.82, 140.95, 144.52. Compound **19**: yield 0.23 g, 3%; mp 107–110 °C; MS (EI, 70 eV): *m/z* 332 (M^+); found: C, 75.69; H, 5.10; N, 8.38. $C_{21}H_{17}N_2$ requires: C, 75.78; H, 5.15; N, 8.42; UV λ_{max} ($\log\varepsilon$): 232.4 (3.68), 244.8 (3.99), 314.6 (4.05); IR (KBr): 1489 (C=N) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.45 (s, 3H), 5.59 (s, 2H), 7.01–7.32 (m, 10H), 7.57 (d, 1H, $J = 8.4$ Hz), 7.74 (d, 1H, $J = 8.7$ Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ 21.17, 53.38, 117.13, 120.23, 121.09, 121.68, 126.25, 126.35, 128.14, 128.63, 129.30, 129.57, 133.38, 135.22, 136.48, 138.89, 148.15.

3.1.4. 1-(3-Chlorobenzyl)-3-(4-methylphenyl)-1*H*-indazole (20) and 2-(3-chlorobenzyl)-3-(4-methylphenyl)-2*H*-indazole (21). Compound **6** (10.4 g, 0.05 mol), EtONa (6.8 g, 0.1 mol), and 3-chlorobenzyl chloride (**9**) (16.1 g, 0.1 mol) were allowed to react as in the preparation of compound **16** and compound **17** to afford compound **20** and compound **21**. Compound **20**: yield 7.1 g, 43%; mp 67–68 °C; MS (EI, 70 eV): *m/z* 332 (M^+); found: C, 75.68; H, 5.09; N, 8.39. $C_{21}H_{17}N_2$ requires: C, 75.78; H, 5.15; N, 8.42; UV λ_{max} ($\log\varepsilon$): 233.2 (3.79), 245.4 (4.09), 313.0 (4.10); IR (KBr): 1492 (C=N) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.32 (s, 3H), 5.48 (s, 2H), 6.98–6.99 (m, 1H), 7.05–7.25 (m, 8H), 7.78 (d, 2H, $J = 8.1$ Hz), 7.92 (d, 1H, $J = 8.1$ Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ 21.30, 52.24, 109.27, 121.09, 121.56, 122.09, 125.20, 126.50, 127.18, 127.37, 127.87, 129.49, 129.95, 130.57, 134.53, 137.80, 138.93, 140.97, 144.56. Compound **21**: yield 0.76 g, 5%; mp 98–100 °C; MS (EI, 70 eV): *m/z* 332 (M^+); found: C, 75.72; H, 5.13; N, 8.41. $C_{21}H_{17}N_2$ requires: C, 75.78; H, 5.15; N, 8.42; UV λ_{max} ($\log\varepsilon$): 232.2 (3.70), 244.2 (4.00), 313.4 (4.04); IR (KBr): 1500 (C=N) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.37 (s, 3H), 5.51 (s, 2H), 6.86–7.29 (m, 10H), 7.50 (dd, 1H, $J = 1.0$, 8.4 Hz), 7.67 (dd, 1H, $J = 0.9$, 8.7 Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ 21.36, 53.64, 117.36, 120.44, 121.26, 121.91, 125.08, 126.39, 126.58, 127.10, 127.95, 129.51, 129.78, 129.96, 134.59, 136.79, 138.84, 139.13, 148.37.

3.1.5. 1-(2-Chlorobenzyl)-3-(4-methylphenyl)-1*H*-indazole (22) and 2-(2-chlorobenzyl)-3-(4-methylphenyl)-2*H*-indazole (23). Compound **6** (10.4 g, 0.05 mol), EtONa (6.8 g, 0.1 mol), and 2-chlorobenzyl chloride (**10**) (16.1 g, 0.1 mol) were allowed to react as in the preparation of compound **16** and compound **17** to afford compound **22** and compound **23**. Compound **22**: yield 7.3 g, 44%; mp 78–79 °C; MS (EI, 70 eV): *m/z* 332 (M^+); found: C, 75.57; H, 4.79; N, 8.49. $C_{21}H_{17}N_2$ requires: C, 75.78; H, 5.15; N, 8.42; UV λ_{max} ($\log\varepsilon$): 232.4 (3.65), 245.4 (3.94), 312.6 (3.99); IR (KBr): 1499 (C=N) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.35 (s, 3H), 5.69 (s, 2H), 6.70–7.35 (m, 9H), 7.81 (d, 2H, $J = 8.1$ Hz), 7.96 (d, 1H, $J = 8.1$ Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ 21.30, 50.00, 109.44, 121.12, 121.50, 121.94, 126.52, 127.10, 127.37, 128.31, 128.75, 129.34, 129.49, 130.66, 132.24, 134.67, 137.79, 141.32, 144.69. Compound **23**: yield 0.58 g, 4%; mp 132–135 °C; MS (EI, 70 eV): *m/z* 332 (M^+); found: C, 75.74; H, 5.10; N, 8.38. $C_{21}H_{17}N_2$ requires: C, 75.78; H, 5.15; N, 8.42; UV λ_{max} ($\log\varepsilon$): 232.8 (3.69), 244.6 (4.00), 314.8 (4.06); IR (KBr): 1503 (C=N) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.43 (s, 3H), 5.74 (s, 2H), 6.63–7.40 (m, 10H), 7.64 (d, 1H, $J = 8.5$ Hz), 7.76 (d, 1H, $J = 8.7$ Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ 21.33, 51.95, 117.43, 120.55, 121.12, 121.94, 126.33, 126.57, 127.28, 127.84, 128.78, 129.19, 129.20, 129.80, 131.73, 135.04, 137.23, 139.01, 148.56.

3.1.6. 1-(4-Methylbenzyl)-3-(4-methylphenyl)-1*H*-indazole (24) and 2-(4-methylbenzyl)-3-(4-methylphenyl)-2*H*-indazole (25). Compound **6** (4.16 g, 0.02 mol), EtONa (2.72 g, 0.04 mol), and 4-methylbenzyl chloride (**11**) (6.3 g, 0.045 mol) were allowed to react as in the preparation of compound **16** and compound **17** to afford compound **24** and compound **25**. Compound **24**: yield 3.1 g, 50%; mp 80–83 °C; MS (EI, 70 eV): *m/z* 312 (M^+); found: C, 84.55; H, 6.41; N, 8.93. $C_{22}H_{20}N_2$ requires: C, 84.58; H, 6.45; N, 8.97; UV λ_{max} ($\log\varepsilon$): 232.8 (3.80), 243.4 (4.09), 313.8 (4.09); IR (KBr): 1490 (C=N) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.21 (s, 3H), 2.34 (s, 3H), 5.53 (s, 2H), 6.99–7.26 (m, 9H), 7.80 (d, 2H, $J = 8.1$ Hz), 7.92 (d, 1H, $J = 8.1$ Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ 21.07, 21.31, 52.85, 109.62, 120.86, 121.44, 122.10, 126.22, 127.14, 127.38, 129.32, 129.46, 130.83, 133.90, 137.34, 137.62, 140.95, 144.10. Compound **25**: yield 0.4 g, 6%; mp 108–110 °C; MS (EI, 70 eV): *m/z* 312 (M^+); found: C, 84.48; H, 6.39; N, 8.88. $C_{22}H_{20}N_2$ requires: C, 84.58; H, 6.45; N, 8.97; UV λ_{max} ($\log\varepsilon$): 33.8 (3.72), 243 (4.04), 266.0 (3.88), 314.6 (4.06); IR (KBr): 1500 (C=N) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.31 (s, 3H), 2.45 (s, 3H), 5.60 (s, 2H), 6.98–7.39 (m, 10H), 7.48 (dd, 1H, $J = 1.0$, 8.4 Hz), 7.66 (dd, 1H, $J = 0.9$, 8.7 Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ 21.06, 21.34, 54.03, 117.39, 120.40, 121.28, 121.62, 126.23, 126.73, 126.90, 129.30, 129.62, 133.98, 136.49, 137.32, 138.83, 148.29.

3.1.7. 1-(3-Methylbenzyl)-3-(4-methylphenyl)-1*H*-indazole (26) and 2-(3-methylbenzyl)-3-(4-methylphenyl)-2*H*-indazole (27). Compound **6** (4.16 g, 0.02 mol), EtONa (2.72 g, 0.04 mol), and 3-methylbenzyl chloride (**12**) (6.3 g, 0.045 mol) were allowed to react as in the preparation of compound **16** and compound **17** to afford compound **26** and compound **27**. Compound **26**: yield 3.2 g, 52%; mp 79–80 °C; MS (EI, 70 eV): *m/z* 312 (M^+); found: C, 84.56; H, 6.42; N, 8.94. $C_{22}H_{20}N_2$ requires: C, 84.58; H, 6.45; N, 8.97; UV λ_{max} ($\log\varepsilon$): 233.2 (3.77), 243.8 (4.06), 314.0 (4.07); IR (KBr): 1490 (C=N) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.19 (s, 3H), 2.34 (s, 3H), 5.52 (s, 2H), 6.95–7.26 (m, 9H), 7.81 (d, 2H, $J = 8.0$ Hz), 7.93 (d, 1H, $J = 8.1$ Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ 21.33, 53.02, 109.61, 120.88, 121.44, 122.07, 124.23, 126.24, 127.39, 127.84, 128.43, 128.53, 129.46, 130.83, 136.84, 137.63, 138.36, 141.01, 144.12. Compound **27**: yield 0.18 g, 3%; mp 79–80 °C; MS (EI, 70 eV): *m/z* 312 (M^+); found: C, 84.54; H, 6.43; N, 9.00. $C_{22}H_{20}N_2$ requires: C, 84.58; H, 6.45; N, 8.97; UV λ_{max} ($\log\varepsilon$): 233.6 (3.76), 243.2 (4.05), 314.3 (4.06); IR (KBr): 1494 (C=N) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.20 (s, 3H), 2.39 (s, 3H),

5.59 (s, 2H), 6.76 (d, 2H, J = 7.4 Hz), 6.86 (s, 2H), 7.02–7.25 (m, 3H), 7.29–7.32 (m, 1H), 7.37 (d, 2H, J = 8.4 Hz), 7.43 (d, 2H, J = 8.4 Hz), 7.52 (d, 2H, J = 8.4 Hz), 7.64 (d, 2H, J = 8.7 Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ 21.17, 53.95, 117.31, 120.45, 120.90, 121.87, 124.21, 126.30, 127.71, 128.41, 128.70, 129.53, 130.02, 135.91, 137.28, 137.92, 138.76, 147.73.

3.1.8. 1-(2-Methylbenzyl)-3-(4-methylphenyl)-1*H*-indazole (28) and 2-(2-methylbenzyl)-3-(4-methylphenyl)-2*H*-indazole (29). Compound **6** (4.16 g, 0.02 mol), EtONa (2.72 g, 0.04 mol), and 2-methylbenzyl chloride (**13**) (6.3 g, 0.045 mol) were allowed to react as in the preparation of compound **16** and compound **17** to afford compound **28** and compound **29**. Compound **28**: yield 3.05 g, 49%; mp 114–117 °C; MS (EI, 70 eV): m/z 312 (M^+); found: C, 84.57; H, 6.41; N, 8.95. $C_{22}H_{20}N_2$ requires: C, 84.58; H, 6.45; N, 8.97; UV λ_{max} (log ε): 33.2 (3.81), 244.8 (4.09), 314.2 (4.12); IR (KBr): 1491 (C=N) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.33 (s, 3H), 2.34 (s, 3H), 5.57 (s, 2H), 6.73 (d, 1H, J = 7.3 Hz), 6.97–7.25 (m, 9H), 7.80 (dd, 2H, J = 1.8, 8.2 Hz), 7.95 (dd, 1H, J = 1.0, 8.1 Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ 19.35, 21.31, 51.27, 109.61, 120.92, 121.49, 121.96, 126.20, 126.28, 127.30, 127.36, 127.63, 129.47, 130.42, 130.78, 134.90, 135.70, 137.67, 141.28, 144.14. Compound **29**: yield 0.28 g, 5%; mp 125–128 °C; MS (EI, 70 eV): m/z 312 (M^+); found: C, 84.49; H, 6.41; N, 8.91. $C_{22}H_{20}N_2$ requires: C, 84.58; H, 6.45; N, 8.97; UV λ_{max} (log ε): 232.6 (3.69), 243.4 (3.99), 315.2 (4.10); IR (KBr): 1493 (C=N) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.16 (s, 3H), 2.35 (s, 3H), 5.54 (s, 2H), 6.53 (d, 1H, J = 7.3 Hz), 6.98–7.30 (9H, m), 7.55 (d, 1H, J = 8.4 Hz), 7.67 (d, 1H, J = 8.7 Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ 19.07, 21.33, 52.21, 117.46, 120.45, 121.14, 121.73, 126.30, 126.44, 126.67, 127.46, 129.27, 129.71, 130.10, 134.54, 135.46, 136.84, 138.85, 148.41.

3.1.9. 1-(4-Methoxylbenzyl)-3-(4-methylphenyl)-1*H*-indazole (30) and 2-(4-methoxylbenzyl)-3-(4-methylphenyl)-2*H*-indazole (31). Compound **6** (10.4 g, 0.05 mol), EtONa (6.8 g, 0.1 mol), and 4-methoxylbenzyl chloride (**14**) (23.5 g, 0.15 mol) were allowed to react as in the preparation of compound **16** and compound **17** to afford compound **30** and compound **31**. Compound **30**: yield 7.3 g, 45%; mp 66–69 °C; MS (EI, 70 eV): m/z 328 (M^+); found: C, 80.43; H, 6.11; N, 8.50. $C_{22}H_{20}N_2$ requires: C, 80.46; H, 6.14; N, 8.53; UV λ_{max} (log ε): 232.8 (4.80), 243.4 (4.10), 313.8 (4.09); IR (KBr): 1512 (C=N) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.31 (s, 3H), 3.63 (s, 3H), 5.59 (s, 2H), 6.81 (d, 2H, J = 8.6 Hz), 7.18 (dd, 1H, J = 7.5, 7.5 Hz), 7.24 (d, 2H, J = 8.6 Hz), 7.28 (d, 2H, J = 8.0 Hz), 7.38 (dd, 1H, J = 7.6, 7.6 Hz), 7.68 (d, 1H, J = 8.6 Hz), 7.87 (d, 2H, J = 8.0 Hz), 8.00 (d, 1H, J = 8.2 Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ 21.12, 51.76, 55.26, 110.53, 114.20, 121.28, 121.35, 121.51, 126.61, 127.08, 129.16, 129.60, 129.80, 130.82, 137.51, 140.98, 143.02, 159.02. Compound **31**: yield 0.49 g, 3%; mp 114–115 °C; MS (EI, 70 eV): m/z 328 (M^+); found: C, 80.42; H, 6.13; N, 8.51. $C_{22}H_{20}N_2$ requires: C, 80.46; H, 6.14; N, 8.53; UV λ_{max} (log ε): 232.8 (3.76), 243.0 (4.08), 313.8 (4.06); IR (KBr): 1512 (C=N) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.37 (s, 3H), 3.66 (s, 3H), 5.50 (s, 2H), 6.80 (d, 2H, J = 8.7 Hz), 6.97 (d, 2H, J = 8.7 Hz), 7.03 (dd, 1H, J = 7.5, 7.5 Hz), 7.26 (dd, 1H, J = 8.0, 8.0 Hz), 7.35 (d, 2H, J = 8.1 Hz), 7.40 (2H, d, J = 8.2 Hz), 7.49 (d, 1H, J = 8.5 Hz), 7.62 (d, 1H, J = 8.7 Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ 21.15, 53.47, 55.27, 114.13, 117.28, 120.41, 120.96, 121.85, 126.28, 126.41, 128.65, 129.26, 129.57, 130.05, 135.69, 138.74, 147.70, 158.87.

3.1.10. 1-(3-Methoxylbenzyl)-3-(4-methylphenyl)-1*H*-indazole (32) and 2-(3-methoxylbenzyl)-3-(4-methylphenyl)-2*H*-indazole (33). Compound **6** (6.24 g, 0.03 mol), EtONa (4.08 g, 0.06 mol), and 3-methoxylbenzyl chloride (**15**) (10.18 g, 0.065 mol) were allowed to react as in the preparation of compound **16** and compound **17** to afford compound **32** and compound **33**. Compound **32**: yield 4.37 g, 44.5%; mp 50–51 °C; MS (EI, 70 eV): m/z 328 (M^+); found: C, 80.49; H, 6.11; N, 8.49. $C_{22}H_{20}N_2$ requires: C, 80.46; H, 6.14; N, 8.53; UV λ_{max} (log ε): 243.2 (3.97), 283.6 (3.93), 314.1 (4.05); IR (KBr): 1493 (C=N) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.36 (s, 3H), 3.68 (s, 3H), 5.67 (s, 2H), 6.78–6.86 (m, 3H), 7.16–7.24 (m, 2H), 7.32 (d, 2H, J = 8.1 Hz), 7.38–7.45 (m, 1H), 7.74 (d, 1H, J = 8.5 Hz), 7.87 (d, 2H, J = 8.1 Hz), 8.05 (d, 1H, J = 8.2 Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ 21.08, 51.99, 55.21, 110.49, 112.79, 113.58, 119.65, 121.18, 121.24, 121.49, 126.61, 126.99, 129.73, 129.94, 130.66, 137.44, 139.23, 141.13, 143.02, 159.56. Compound **33**: yield 0.39 g, 4%; mp 98–100 °C; MS (EI, 70 eV): m/z 328 (M^+); found: C, 80.39; H, 6.06; N, 8.50. $C_{22}H_{20}N_2$ requires: C, 80.46; H, 6.14; N, 8.53; UV λ_{max} (log ε): 243.0 (3.99), 283.2 (3.91), 314.0 (4.05); IR (KBr): 1489 (C=N) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.37 (s, 3H), 3.64 (s, 3H), 5.52 (s, 2H), 6.57–7.30 (m, 10H), 7.50 (d, 1H, J = 8.1 Hz), 7.66 (d, 1H, J = 8.3 Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ 21.31, 54.15, 55.12, 112.55, 113.14, 117.38, 119.17, 120.40, 121.25, 121.68, 126.30, 126.65, 129.55, 129.66, 136.58, 138.50, 138.86, 148.33, 159.79.

3.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated according to the protocols from Jaffe et al.[11], obtained from human umbilical cord veins with collagenase, and cultured in 75 cm^2 plastic flasks in M199 containing 20% FBS, 15 μg/mL endothelial cell growth supplements (ECGs). Confirmation of their identity as endothelial cells was provided by detection of CD31 (PECAM-1), as assessed by immunostaining. Experiments were conducted on HUVECs that had been used in passage 2–5.

3.3. [^{3}H]Thymidine incorporation assay

Confluent HUVECs were trypsinized, suspended in DMEM supplemented with 20% FBS, and seeded at 1.0×10^4 cells per well into 96-well plates. After 24 h, the cells were washed twice with PBS and starved with 2% FBS-M199 medium for 24 h. The cells were incubated with or without indicated reagents and growth fac-

tors (VEGF; 10 ng/mL) for 24 h and harvested. Before the harvest, cells were incubated with [^{3}H]thymidine (2 μCi/mL) for 4 h, harvested with Filter-Mate (Packard), and incorporated radioactivity was determined.

3.4. Cytotoxicity

3.4.1. Reagents. RPMI-1640 medium, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from GIBCO BRL (Grand Island, NY, USA). 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

3.4.2. Cell line and cell culture. Human leukemia HL-60 cells were obtained from ATCC. Cells were cultured in RPMI-1640 medium supplemented with 10% FBS, penicillin (100 units/mL)/streptomycin (10 μg/mL), and 1% L-glutamine at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were split every day to maintain the cell numbers between 2 and 5×10^5 cells/mL. The cell numbers were assessed by the standard procedure of leukocyte counting using a hemocytometer.

3.4.3. Anti-proliferative analysis of HL-60 cells and human normal leukocytes. HL-60 cells or human normal leukocytes were seeded at a density of 1×10^5 cells/mL in 24-well culture plates and treated with test compounds or vehicle for 24 or 48 h. All of the test compounds were dissolved in DMSO, and the final concentration of DMSO in the culture medium was kept below 0.1%. The anti-proliferative effect was assessed using the MTT assay. We briefly added 10 μL MTT solution (5 mg/mL) with 50 μL cell suspension in HBSS into a 96-well plate and incubated at 37 °C in the dark for 2 h. Treatment of living cells with MTT produces a dark blue formazan product, whereas no such staining is observed in dead cells. The formazan product was dissolved by adding 150 μL DMSO and then the absorbance was measured on an ELISA reader at a best wavelength of 570 nm.

3.5. In vivo Matrigel plug assay

Nude mice (6 weeks of age) were given sc injections of 500 μL of Matrigel (Becton Dickinson, Bedford, MA) at 4 °C with or without **YD-3** analogues and growth factor (150 ng/mL VEGF). After injection, the Matrigel rapidly formed a plug. After 7 days, the skin of the mouse was easily pulled back to expose the Matrigel plug, which remained intact. After quantitative differences were noted and photographed, hemoglobin was measured, as an indication of blood vessel formation, using the Drabkin method (Drabkin reagent kit 525, Sigma, St. Louis, MO). The concentration of hemoglobin was calculated from a known amount of hemoglobin assayed in parallel.

Acknowledgment

The investigation was supported by a research grant from the National Science Council of the Republic of China (NSC 91-2320-B-039-025) awarded to S. C. Kuo.

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