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Synthesis and antiangiogenic activity of 6-amido-2,4,5-trimethylpyridin-3-ols

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ABSTRACT

We recently reported that 6-aminoalkyl-2,4,5-trimethylpyridin-3-ols, novel series of 6-aminopyridin-3-ol-based antioxidants, have high antiangiogenic activities. In pursuit of wider variety in the analogues, we here report the synthesis and antiangiogenic activities of 6-amidoalkyl-2,4,5-trimethylpyridin-3-ols, which would not be considered excellent antioxidants because of the poorer electron-donating effect of the C(6)-amido group than the corresponding C(6)-amino group. The selected 6-amido compounds showed up to several fold-higher antiangiogenic activities and up to an order of magnitude better antitumor activities in the chick embryo chorioallantoic membrane (CAM) assay than SU4312, a positive control. We also found that paracetamol, as a direct phenolic analogue of our simplest 6-amidopyridin-3-ol, showed a moderate level of antiangiogenic activity. We propose this study will offer a basis for a scaffold of novel angiogenesis inhibitors that can perturb angiogenesis-related pathologies.

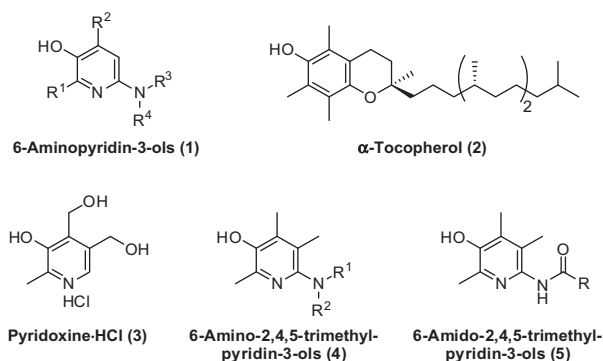
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Angiogenesis is a biological process where new blood vessels branch out from the existing vasculature.¹ This process is tightly controlled for normal physiologies such as reproduction, development, and wound repair. However, it becomes out of control when it is implicated in diseases such as cancer, age-related or diabetic retinopathy and so on.² For example, angiogenesis is significantly increased around solid tumor tissues which require a high level of nutrients and oxygen to grow to a considerable size. New blood vessels, in turn, enable cancer cells to invade adjacent tissues and to migrate throughout the body (metastasis). Significant efforts have been made to develop angiogenesis inhibitors, which block tumor angiogenesis, for the prevention and treatment of cancer.³ Synthetic antiangiogenic drugs include sorafenib and sunitinib which were approved by the FDA for the treatment of cancer.⁴ Various types of angiogenesis inhibitors are currently being tested in clinical trials.⁵ For example, thalidomide, once a tragic drug, has recently received a great deal of attention as an antitumor agent due to its excellent antiangiogenic activities.⁶

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6-Aminopyridin-3-ol (1) has been known as a novel antioxidant scaffold for years, featuring enhanced air-stability by virtue of the ring nitrogen and potent antioxidant activity toward lipid peroxy radical.⁷ Mono- and bicyclic analogues with lipophilic side chains showed higher levels of antioxidant activity and binding affinity (k_d) to tocopherol transfer protein (TPP) than α -tocopherol (2), the most effective natural lipophilic antioxidant.^{7d-g} Also, they protect the endogenous α -tocopherol from oxidative consumption and do not participate in tocopherol mediated peroxidation process.^{7d,e}

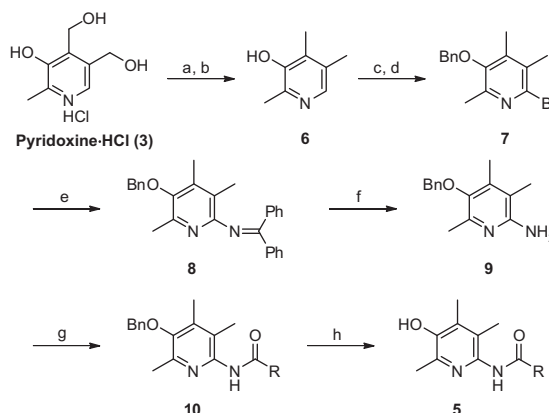
We recently reported 6-aminoalkyl-2,4,5-trimethylpyridin-3-ols (**4**, R^1 or R^2 = H or alkyl) as novel series of 6-aminopyridin-3-ol antioxidants.^{7f,g} They were found to be good antioxidants with potent radical-scavenging activities. A lipophilic analogue (i.e., R^1 = n -C₁₆H₃₃, R^2 = H in **4**) showed greater capacity to prevent plasma lipid peroxidation and higher efficacy to protect cells from oxidative injury than α -tocopherol. A hydrophilic, water-soluble analogue (i.e., R^1 = R^2 = H in **4**) acted as a co-antioxidant in a heterogeneous system that may serve to extend the half-life of α -tocopherol. Another profound feature of this series is the facile synthetic method starting from pyridoxine hydrochloride (**3**) which is readily available in quantity.

Based on the notion that the electron donating groups at *ortho*- and *para*-positions to —OH group can decrease bond dissociation enthalpy of O—H bond of phenolic antioxidants,⁸ thereby increasing H atom transfer to lipid peroxy radicals, C(6)-amino functions of 6-aminopyridin-3-ols (**1** and **4**) have almost always been mono- or dialkylated or arylated in pursuit of high H-atom donation ability as chain-breaking antioxidants. In parallel, studies of their biological activities have focused largely on the antioxidant activities to neutralize reactive oxygen species (ROS). There are only a few reports on their pharmacological actions against disease-related systems.⁹

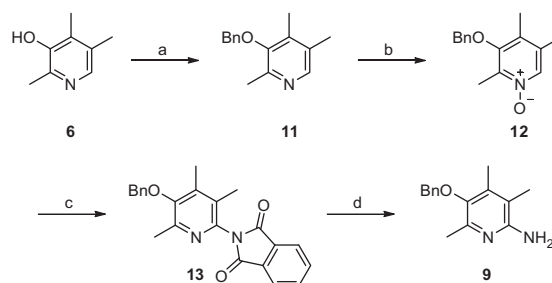
However, we recently found for the first time that 6-alkylamino-2,4,5-trimethylpyridin-3-ols (**4**) have very potent antiangiogenic activity in the chick embryo chorioallantoic membrane (CAM) assay.¹⁰ The best analogues showed more than 3-fold better ID₅₀ than SU4312, a well-known angiogenesis inhibitor.¹¹ They also significantly inhibited angiogenic cancer-formation in a cancer cell-inoculated CAM assay. Interestingly, we have not observed a particularly good correlation between antiangiogenic activities and antioxidant activities of 6-alkylamino-2,4,5-trimethylpyridin-3-ols. There was no meaningful distinction in antiangiogenic activities between the analogues with an electron donation group and the ones with an electron withdrawing group on C(6)-amino moiety. In fact, the best antiangiogenic analogues include the one with *p*-nitrophenyl group attached to C(6)-amino group.

Inspired by this observation, we introduced 6-amido groups in place of 6-amino groups, which would be poor substituents for antioxidants, because they will cause the pyridinols to be electron poorer and presumably less effective antioxidants. We report, in fact, that 6-amido analogues **5**, in general, have improved antiangiogenic and antitumor activities compared to 6-amino analogues **4**. We also report that paracetamol, which is a well-known active ingredient of an antipyretic and analgesic drug, as a direct phenolic analogue of our simplest 6-amidopyridin-3-ol, showed moderate antiangiogenic activity. We propose that this study will offer the basis for a scaffold of novel angiogenesis inhibitors that can perturb angiogenesis-related pathologies.

Shown in Scheme 1 is a synthesis of 6-amido-2,4,5-trimethylpyridin-3-ol analogues (**5**) starting from pyridoxine hydrochloride (**3**). Our initial synthetic pathway utilized 6-amino-3-benzyloxy-2,4,5-trimethylpyridine (**9**) as a key intermediate. Briefly, two primary alcohols of **3** were reductively removed via chlorides to afford two methyl groups of **6**. C(6)-position and C(3)-OH of **6** were brominated and benzylated, respectively, in consecutive manner, to afford **7**. Then, for the installation of amino group, benzophenone imine, as an ammonia equivalent, was subjected to Buchwald–Hartwig amination condition to afford a C(6)-benzophenone imine-installed derivative **8** in 83% yield. It was then hydrolyzed to conveniently give **9** in 83% yield.¹⁰ Various acid chlorides were reacted with **9** to give the amides **10** which were next hydrogenolyzed to afford 6-amido-2,4,5-trimethylpyridin-3-ols (**5**).



Scheme 1. Synthesis of 6-amido-2,4,5-trimethylpyridin-3-ols. Reagents and conditions: (a) SOCl₂, DMF (cat.), reflux, 30 min, 93%; (b) Zn, AcOH, reflux, 3 h, 92%; (c) DBDMH, THF, rt, 3 h, 80%; (d) PhCH₂Cl, K₂CO₃, DMF, rt, 12 h, 97%; (e) benzophenone imine, Pd₂(dba)₃, BINAP, NaO^tBu, toluene, reflux, 12 h, 83%; (f) HCl, MeOH–THF, rt, 12 h, 83%; (g) acyl chloride, Et₃N, CH₂Cl₂, rt; (h) H₂, Pd/C, MeOH, rt or BCl₃, CH₂Cl₂, 0 °C (for **55**).



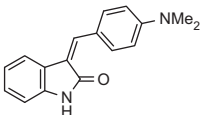
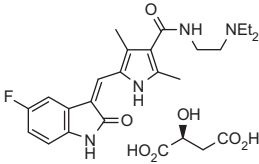
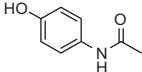
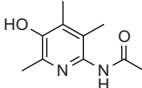
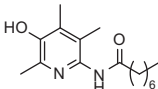
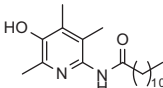
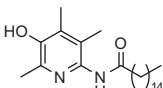
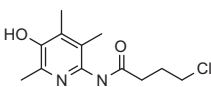
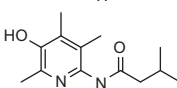
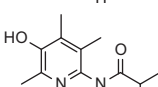
Scheme 2. An alternative synthesis of 6-amino-3-benzyloxy-2,4,5-trimethylpyridin-3-ol (**9**). Reagents and conditions: (a) PhCH₂Cl, K₂CO₃, DMF, rt, 12 h, 71%; (b) *m*-CPBA, CH₂Cl₂, rt, 1 h, 94%; (c) *p*-TsCl, *i*-Pr₂NEt, phthalimide, CH₂Cl₂, rt, 12 h, 70%; (d) H₂NNH₂, THF–EtOH, rt, 1 h, 90%.

In an alternative approach shown in Scheme 2, the amino group of **10** was introduced through a slightly different pathway. First, C(3)-OH of compound **6** was benzylated to afford **11** in 71% yield. Then, the nitrogen of the pyridine ring of **11** was oxidized to an *N*-oxide with *m*-CPBA to give **12** in 94% yield. Subsequently, the treatment of **12** with *p*-toluenesulfonyl chloride and phthalimide in the presence of Hünig's base gave **13** in 70% yield,¹² which was finally converted to the amine **9** in 90% yield by a simple treatment with hydrazine.

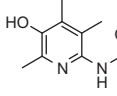
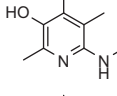
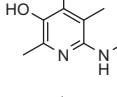
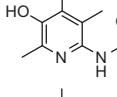
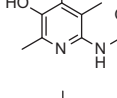
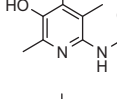
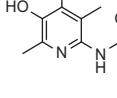
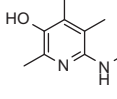
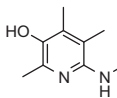
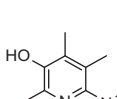
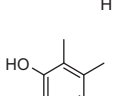
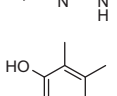
A variety of amide groups were introduced in **5** as R groups shown in Scheme 1. R groups range from various alkyl chains with various lengths, cycloalkanes, and aromatic rings with various types of methylene spacers, electron donating groups, electron withdrawing groups, and heteroatoms.

Angiogenesis inhibitory activities of 6-amido-2,4,5-trimethylpyridin-3-ols (**5**) were evaluated using quantitative CAM assay,¹³ which is one of the most useful in vivo assay models of angiogenesis.¹⁴ In CAM assays, angiogenesis was induced by treating CAMs with vascular endothelial growth factor (VEGF), the best characterized proangiogenic factor, prior to the compound treatment. Then, test compounds soaked in a disk were treated on CAM. As shown in Table 1, treatment with VEGF (20 ng/CAM) significantly enhanced the number of newly formed blood vessel branch points compared to phosphate buffered saline (PBS)-treated control group. At a fixed

Inhibitory effects of 6-amido-2,4,5-trimethylpyridin-3-ols (**5A-S**) on VEGF-induced angiogenesis in vivo^a

Compound	Structure	Inhibition (%) at 0.01 nmol/CAM ^b
SU4312		78.0 ± 4.8 ^a
Sunitinib malate		88.8 ± 8.1 ^a
Paracetamol		77.9 ± 10.6 ^a
5A		84.8 ± 10.0 ^a
5B		74.7 ± 4.6 ^a
5C		57.4 ± 10.0 ^a
5D		59.9 ± 11.3 ^a
5E		70.2 ± 4.6 ^a
5F		75.6 ± 8.9 ^a
5G		53.2 ± 11.5 ^a

Compound	Structure	Inhibition (%) at 0.01 nmol/CAM ^b
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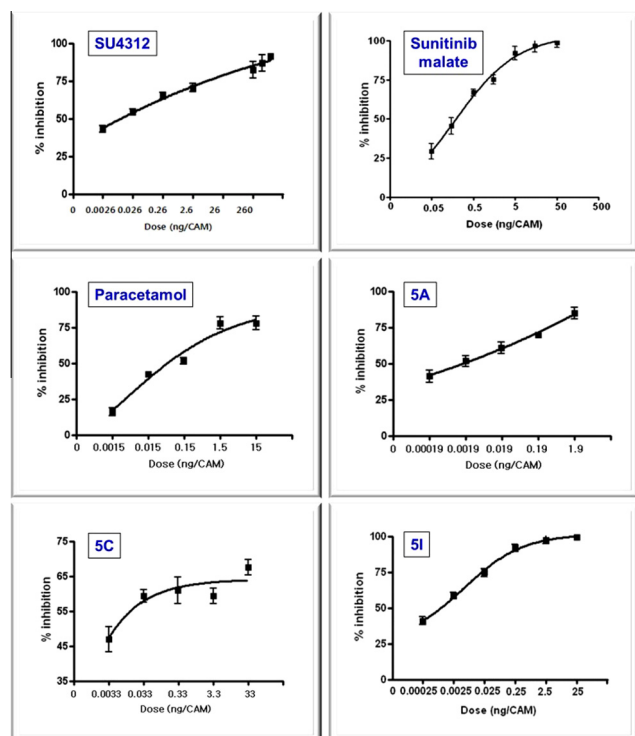
Compound	Chemical Structure	IC ₅₀ (μM)
5H		73.2 ± 6.4 [*]
5I		81.8 ± 8.4 [*]
5J		76.3 ± 7.2 [*]
5K		69.6 ± 11.0 [*]
5L		74.2 ± 3.9 [*]
5M		77.3 ± 13.1 [*]
5N		75.3 ± 6.3 [*]
5O		69.7 ± 4.5 [*]
5P		74.2 ± 7.0 [*]
5Q		64.8 ± 17.4 [*]
5R		75.8 ± 5.4 [*]
5S		74.8 ± 13.2 [*]

^a Quantification of new branches formed from existing blood vessels was performed. Photographs were imported into an Image software program, to measure the new branch points. The data is expressed as the mean \pm SEM of at least six chick embryos. Dose was 0.01 nmol/CAM for both test compounds and SU4312, which is equivalent to 2–3 ng/CAM depending on the molecular weights.

^b Inhibition (%) = [(the number of vessel branch points induced by VEGF) - (the number of vessel branch points induced by test compound and VEGF)] / [(the number of vessel branch points induced by VEGF)] × 100.

* $P < 0.05$ compared to the VEGF-treated CAM sample.

Table 2
Dose-response curves and ID₅₀ values of the selected compounds^a



Compound	ID ₅₀ (pg/CAM)	ID ₅₀ (fmol/CAM)
SU4312	10.5	39.7
Sunitinib malate	202.4	380.0
Paracetamol	60.0	396.9
5A	1.7	8.7
5C	5.0	14.9
5I	0.9	3.6

^a Each data point represents mean \pm SEM (*n*, at least six) at indicated compound doses. ID₅₀'s were calculated using Prism (GraphPad software).

dose of 0.01 nmol/CAM,¹⁵ the 6-amido-2,4,5-trimethylpyridin-3-ols (**5A–S**) significantly suppressed the VEGF-induced angiogenesis, with compound **5A** being the most effective one and compound **5G** being the least effective one among the analogues.

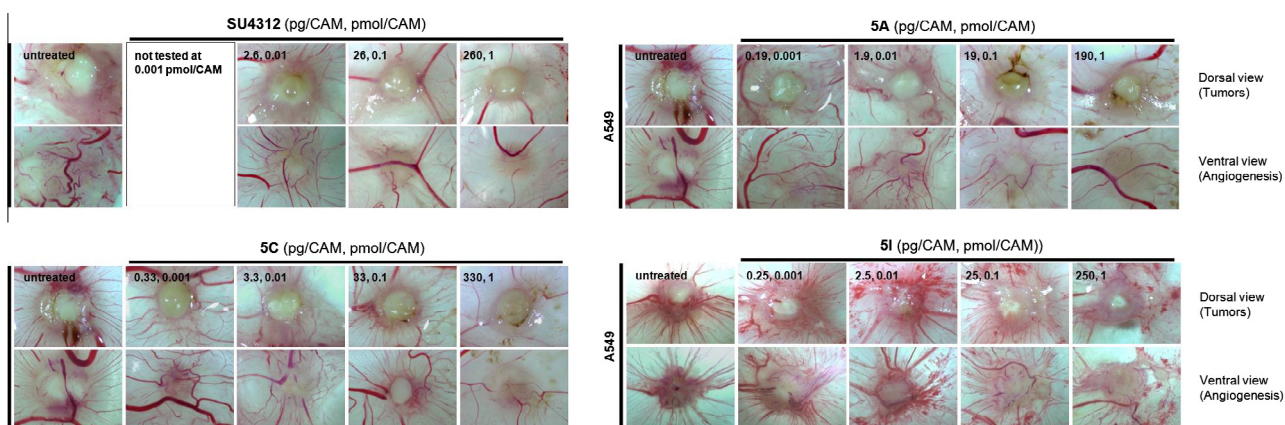
SU4312 and sunitinib were used as positive controls. SU4312 is known as a selective and potent VEGF-receptor (VEGFR) tyrosine kinase inhibitor,^{11a–c} whereas sunitinib, treated as its malate salt in this study, is a multi-targeted receptor tyrosine kinase inhibitor preventing the signalling of VEGFR, platelet-derived growth factor receptor (PDGFR), Kit, colony stimulating factor (CSF)-1R, and RET.^{11d} In general, 6-amidopyridin-3-ols (**5**) showed moderate to high levels of inhibitory activities at a fixed dose (0.01 nmol/CAM). Among them, the simplest methyl (**5A**) and cyclopentyl (**5I**) analogues were particularly strong showing better activities than SU4312. Analogues with aromatic groups (**5K–S**) generally showed strong activities regardless of the existence of methylene spacer(s), electron-donating or withdrawing group(s), heteroaromatic and bicyclic aromatic rings. The analogues with *n*-alkyl substituents (**5A–E**) were also quite strong but showed rather complex structure–activity relationships. The simplest methyl analogue (**5A**) showed the best activity while the longer alkyl analogues gradually lost the activity. However, the analogues with branched alkyls (**5F–H**) and cycloalkanes (**5I**,

5J) restored the activity except for the relatively simple isopropyl (**5G**) substituent.

We then examined dose-dependency and the 50% inhibitory dose (ID₅₀) of **5A**, **5I** (strong ones) and **5C** (weak one), along with paracetamol, in VEGF-induced angiogenesis. Paracetamol can be considered the simplest phenolic analogue of **5A**, from a structural point of view, and can thereby provide some information about the structure–activity relationship. It is a non-selective cyclooxygenase inhibitor and one of the most widely used anti-pyretic and analgesic drugs. It was found to share, in part, a pharmacological mode of action with aminopyridinol by which they inhibit cyclooxygenase- and lipoxygenase-mediated lipid peroxidation.¹⁶ Table 2 displays dose–response curves and remarkable ID₅₀'s of the compounds. The order of ID₅₀ of SU4312, sunitinib malate, and the three selected 6-amidopyridin-3-ols (**5A**, **5C**, and **5I**) is reasonably consistent with the trend found in single-dose data shown in Table 1. In the current study, we report for the first time that SU4312 was more potent inhibitor of *in vivo* angiogenesis using CAM assay than sunitinib malate. Significantly, **5A** and **5I** dose-dependently inhibited angiogenesis and showed more than an order of magnitude better ID₅₀ than SU4312. It is remarkable that paracetamol showed moderate level of antiangiogenic activity as a structural analogue of **5A**, indicating the potential roles of three ring methyl groups and pyridine ring of **5A** in the inhibition of angiogenesis.

Cancer is one of the most serious disease conditions in which angiogenesis is deeply involved.¹⁷ In order to examine if the antiangiogenic activities of our compounds can also affected tumorigenesis, **5A**, **5C**, and **5I** were tested in cancer cell-inoculated CAM assay. Implanted A549 human lung cancer cells in a mixture of Matrigel onto CAM dramatically induced angiogenesis and corresponding tumor growth (untreated condition in Table 3). In contrast, all tested compounds (**5A**, **5C**, **5I**, and SU4312), treated once at the time of implantation, showed potent and dose-dependent inhibition of cancer cell-induced angiogenesis (Table 3), measured by the same methods as Table 1. In line with our hypothesis for the antitumor activity, test compounds that showed a significant level of angiogenesis inhibition also suppressed the growth of tumors. It is notable that more than one order of magnitude lower doses of **5A**, **5C**, and **5I** were required to achieve the same level of antitumor activity than of SU4312 at low dose ranges (i.e., 0.19 and 1.9 pg/CAM for **5A**). At higher dosages (i.e., 19 and 190 pg/CAM for **5A**), they showed up to almost 2-fold better activity than SU4312. The degrees of the reduction in size and weight of the excised tumors shown in Table 3 were proportionate to the degrees of angiogenesis inhibition by each compound. This result strongly suggests that the antitumor activity of **5A**, **5C**, and **5I** was due to the suppression of angiogenesis. It also indicates that other factors besides the electron density of the pyridinol ring, an important property for antioxidant activity, dictate the inhibitory activities against angiogenesis and thus tumor growth. Taken together, the data warrant further studies to define the pharmacophore in more detail and to elucidate the mode of action.

In summary, a new series of 6-amido-2,4,5-trimethylpyridin-3-ols (**5**) were synthesized from pyridoxine-HCl (**3**). In this study, **5I** inhibited VEGF-induced angiogenesis better than SU4312 and sunitinib malate. In particular, compounds **5A** and **5I** showed much better antiangiogenic antitumor activity than SU4312. It indicates that their antitumor activities are correlated with the antiangiogenic activities and that other factors might control the antiangiogenic-based activities besides the electron density of the pyridinol ring. Further studies on the pharmacophore and the mode of action are warranted.

Table 3Antiangiogenic and antitumor effects of compounds **5A**, **5C**, **5I**, and SU4312 on A549 human lung cancer cell-inoculated CAMs^a

Compound	Treatment		Angiogenesis		Tumor		
	pg/CAM	pmol/CAM	Vessel branch points around tumor (No.)	Inhibition (%)	Tumor weight (mg)	Inhibition (%)	Excised tumor
SU4312	Untreated	—	124.2 ± 4.5	—	17.6 ± 1.8	—	
	2.6	0.01	98.4 ± 1.7*	20.8 ± 1.4*	14.8 ± 3.6	16.2 ± 20.4	
	26	0.1	93.0 ± 3.5*	25.1 ± 2.8*	13.5 ± 1.6	23.4 ± 9.3	
	260	1.0	79.2 ± 2.4*	36.2 ± 1.9*	12.9 ± 0.9	26.8 ± 4.9	
5A	Untreated	—	120.7 ± 5.3	—	16.7 ± 1.5	—	
	0.19	0.001	105.4 ± 7.9	12.7 ± 6.5	13.3 ± 1.1	20.3 ± 5.9	
	1.9	0.01	92.0 ± 6.3*	23.8 ± 5.2*	11.0 ± 0.9*	34.2 ± 5.3*	
	19	0.1	84.0 ± 4.4*	30.4 ± 3.7*	10.4 ± 1.2*	37.9 ± 8.0*	
5C	Untreated	—	120.7 ± 5.3	—	16.7 ± 1.5	—	
	0.33	0.001	106.0 ± 5.3	12.2 ± 4.4	13.1 ± 1.4	21.7 ± 8.9	
	3.3	0.01	99.0 ± 3.8*	18.0 ± 3.2*	12.4 ± 1.4	26.0 ± 10.0	
	33	0.1	95.0 ± 2.5*	21.3 ± 2.0*	11.5 ± 1.1*	31.3 ± 7.3*	
5I	Untreated	—	116.5 ± 4.3	—	29.7 ± 1.4	—	
	0.25	0.001	80.5 ± 3.8*	30.9 ± 8.5*	26.3 ± 1.1	13.2 ± 3.5	
	2.5	0.01	60.2 ± 1.8*	48.4 ± 4.1*	23.1 ± 0.5*	24.0 ± 1.6*	
	25	0.1	53.1 ± 3.0*	54.4 ± 6.7*	16.7 ± 1.0*	45.1 ± 3.1*	
	250	1.0	48.8 ± 2.9*	58.1 ± 6.8*	11.3 ± 0.8*	62.7 ± 2.3*	

^a A549 human lung cancer cells (2×10^6 cells/CAM) were inoculated on top of CAM, and different concentrations of **5A**, **5C**, **5I**, and SU4312 were treated. The number of new vessel branches was quantitated by the same method as described in Table 1. Tumor mass grown on top of CAM was isolated and weighed. The data represent the means ± SEM of at least six chick embryos.

* $P < 0.05$ compared to the vehicle-treated group.

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Supplementary data

Supplementary data (experimental details along with ^1H & ^{13}C NMR spectra of all new compounds) associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmcl.2014.05.005>.

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