J|A|C|S ARTICLES

Published on Web 05/26/2009

Discovery of Potent and Practical Antiangiogenic Agents Inspired by Cortistatin A

Barbara Czakó,[†] László Kürti,[†] Akiko Mammoto,[‡] Donald E. Ingber,[‡] and E. J. Corey*,[†]

Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, Massachusetts 02138, and Department of Pathology, Harvard Medical School, Vascular Biology Program, Childrens Hospital, 300 Longwood Avenue, Boston, Massachusetts 02115

Received April 1, 2009; E-mail: corey@chemistry.harvard.edu

Abstract: The discovery that cortistatins A and J show noteworthy antiangiogenic activity prompted an investigation of the possibility that simpler and much more easily made compounds based on a steroid core might have useful bioactivity. These studies have led to the development of several potent, water-soluble compounds that may be suitable for local application to treat ocular wet macular degeneration, an important cause of blindness, as well as for treatment of various other angiogenesis-dependent diseases. One of these substances was tested in a mouse retinal angiogenesis model and found to inhibit angiogenesis at a locally administered dose of 500 pmol. Comparison of cell migration data for this and two other synthetic compounds with published data on cortistatin A indicate that they inhibit vascular endothelial growth factor-induced cell migration of human umbilical vein endothelial cells more strongly than cortistatin A.

Introduction

One of the most significant recent developments in cancer therapy is the discovery of a high-affinity antibody that neutralizes the action of the vascular endothelial growth factor (VEGF).[1] That antibody (Avastin, Genentech) now has sales of several billion dollars per annum. A modification of this recombinant DNA-derived antibody is also useful for the treatment of wet macular degeneration. Marketed as Lucentis by Genentech, it has annual sales of about a billion dollars. In cancer therapy, the antibody functions to inhibit the formation of new blood vessels that are required for solid tumor growth.[1,2] In the case of wet macular degeneration, the antibody inhibits the excessive proliferation of blood vessels that leads to the destruction of healthy tissue and consequently compromises vision.[3]

Our research was stimulated by the discovery of a potent antiangiogenic natural product, cortistatin A (**1**, Figure 1), isolated as a trace component from a marine sponge, *Corticulum simplex*, by Kobayashi in 2006.[4] This complex steroidal natural product was shown to exhibit highly selective antiproliferative activity against human umbilical vein endothelial cells (HUVEC) at nanomolar concentrations. It also was found to inhibit VEGF-induced migration and basic fibroblast growth factor (bFGF)-induced tubular network formation of HUVECs at ca. 200 nM concentrations.[5] In addition to cortistatin A (**1**), 10 related natural products have been isolated from the same sponge, one of which, cortistatin J (**10**), also exhibited good antiangiogenic activity (Figure 1).[6,7] To date, the exact cellular target of the cortistatins has not been determined, and *in vivo* studies of **1** have not been reported.

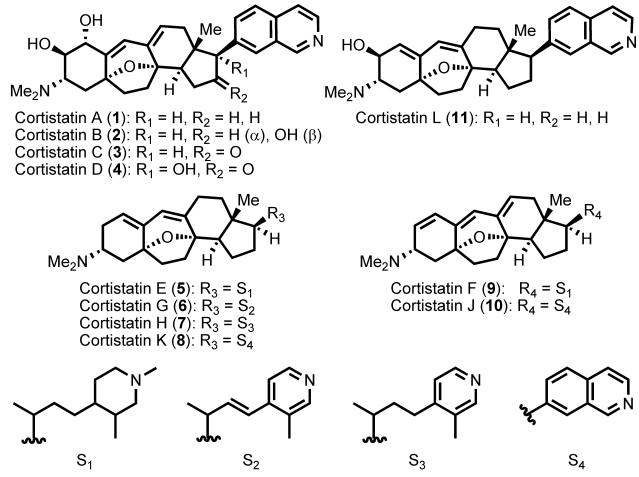


Figure 1. Structures of cortistatin natural products.

Initially we were intrigued by the challenge of devising a synthesis of cortistatin A and carried out preliminary studies

[†] Harvard University.
[‡] Harvard Medical School.

(1) Kim, K. J.; Li, B.; Winer, J.; Armanini, M.; Gillett, N.; Phillips, H. S.; Ferrara, N. *Nature* **1993**, *362*, 841–844.
(2) Folkman, J. *N. Engl. J. Med.* **1971**, *285*, 1182–1186.
(3) Ferrara, N.; Damico, L.; Shams, N.; Lowman, H.; Kim, R. *Retina* **2006**, *26*, 859–870.
(4) Aoki, S.; Watanabe, Y.; Sanagawa, M.; Setiawan, A.; Kotoku, N.; Kobayashi, M. *J. Am. Chem. Soc.* **2006**, *128*, 3148–3149.
(5) Aoki, S.; Watanabe, Y.; Tanabe, D.; Arai, M.; Suna, H.; Miyamoto, K.; Tsujibo, H.; Tsujikawa, K.; Yamamoto, H.; Kobayashi, M. *Bioorg. Med. Chem.* **2007**, *15*, 6758–6762.
(6) Aoki, S.; Watanabe, Y.; Tanabe, D.; Setiawan, A.; Arai, M.; Kobayashi, M. *Tetrahedron Lett.* **2007**, *48*, 4485–4488.
(7) Watanabe, Y.; Aoki, S.; Tanabe, D.; Setiawan, A.; Kobayashi, M. *Tetrahedron* **2007**, *63*, 4074–4079.

10.1021/ja902601e CCC: $40.75

on a synthetic pathway to **1** from the readily available starting material, estrone.[8] As this work progressed, we realized that instead of targeting the total synthesis of **1**, it would be a more important contribution to take the natural product as a lead molecule and initiate a research program to devise and synthesize analogues that have equal or greater biological activity, but are structurally less complex and much more readily available for further biological studies than the natural product. To date, three total syntheses of cortistatin A have been reported. However, only milligram quantities of synthetic **1** have been prepared.[9]

Design and Synthesis of Analogues of Cortistatin A

In order to design and synthesize structurally simple but biologically active analogues of the natural cortistatins, we first identified the minimal structural elements present in the natural products that are essential for antiangiogenic activity. Based on the biological data reported for the various cortistatins, the following conclusions can be drawn:[5] [1] The most active members of the coristatin family, coristatin A (**1**) and cortistatin J (**10**), incorporate a dimethylamino group at the C3 position and an isoquinoline appendage at C17, suggesting that these subunits contribute significantly to biological activity. [2] Cortistatin J (**10**) does not have hydroxyl groups at C1 and C2 positions in contrast to cortistatin A (**1**), implying that these groups may not be essential. [3] Substitution at the C16 and C17 positions is not tolerated and leads to a significant decrease of growth inhibition of HUVECs [coristatins B (**2**) and D (**4**)]. [4] Replacement of the isoquinoline subunit with a 4-isopentyl-1,3-dimethylpiperidine [cortistatins E (**5**) and F (**9**)], a 3-methyl-4-(3-methylbut-1-enyl)pyridine [cortistatin G (**6**)], or a 4-isopentyl-3-methylpyridine side chain [cortistatin H (**7**)] results in decreased biological activity and selectivity.

On the basis of these data and the assumption that the distance between the dimethylamino and isoquinoline substituents should be maintained, it seemed logical to evaluate compounds having a steroidal core, such as **12** (Figure 2). Such structures have the advantage of being synthetically accessible and, hence, potentially practical for therapeutic use. We were attracted to compounds containing a C16−C17 double bond because overlay studies suggested a better fit for this compound than the corresponding saturated derivative,[10] and for ease of synthesis. Our plan also encompassed the study of diastereomeric 3α- and 3*β*-amino compounds and 19-norsteroids, as well as 19-methyl-containing steroids.

Analogues **12** and **13** were prepared starting from the 17-ketal of 3-*O*-methyl estrone **14** using the sequence shown in Scheme 1: Birch reduction of **14**, selective acidic hydrolysis of the resulting enol ether, base-mediated isomerization of the double bond,[11] and Li/NH_3 reduction of the α,*β*-enone provided ketone **15**. Although reductive amination of **15** using

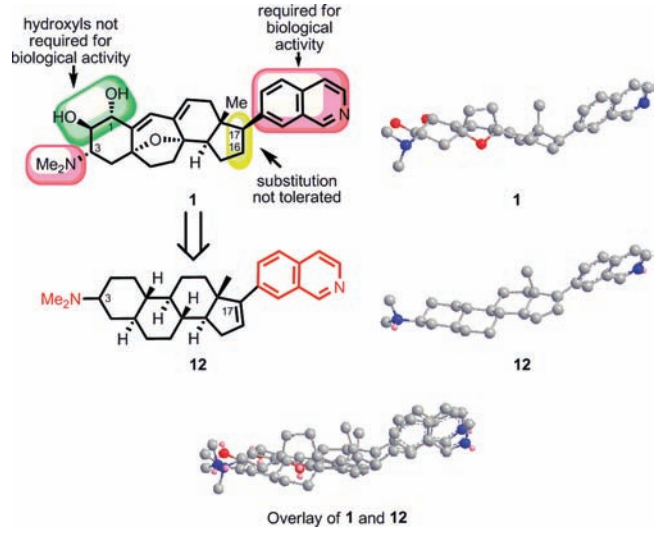
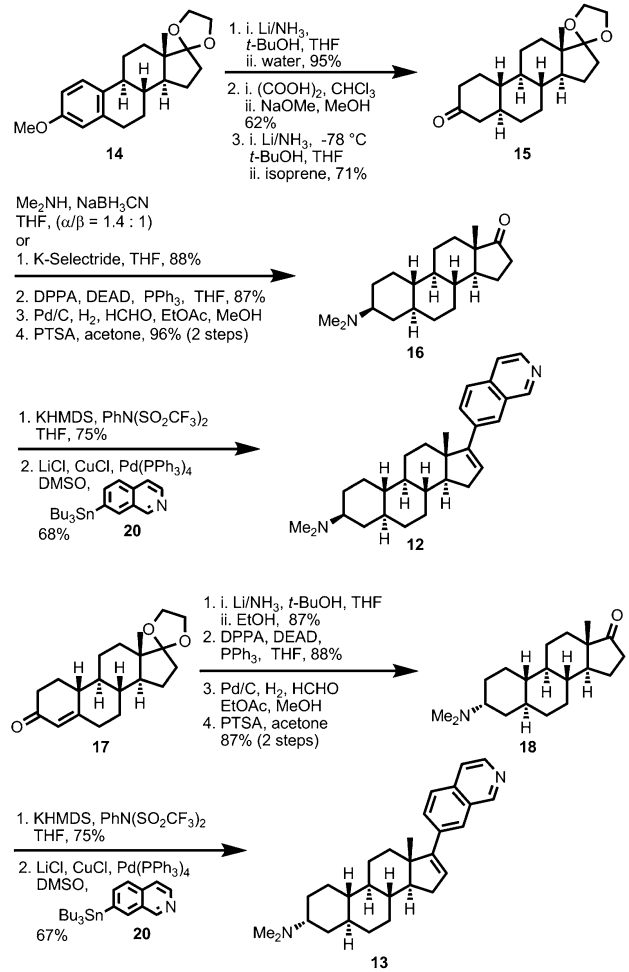


Figure 2. Structurally simple analogue of cortistatin A.

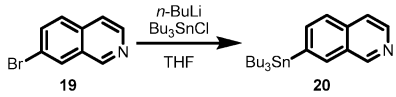
Scheme 1. Synthesis of Analogues **12** and **13**



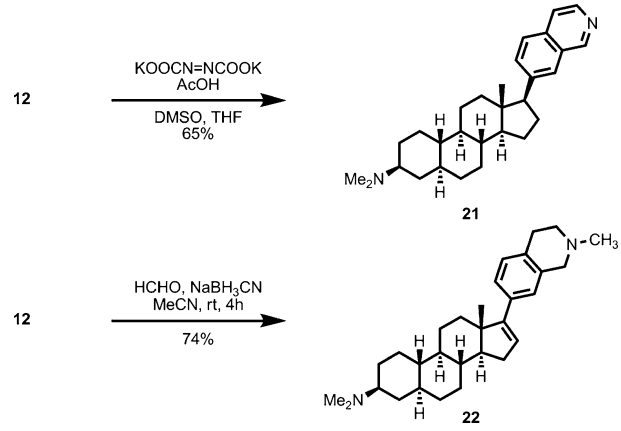
dimethylamine afforded a 1.4:1 mixture of diastereomers, the *β*-dimethylamino ketone **16** could be obtained diastereoselectively via the sequence: (1) reduction of the ketone **15** with K-Selectride, (2) Mitsunobu inversion to the azide, (3) reduction, (4) methylation of the resulting amine, and (5) removal of the ketal protecting group. Subsequently, ketone **16** was converted to the corresponding enol triflate, which

(8) Kürti, L.; Czakó, B.; Corey, E. J. *Org. Lett.* **2008**, *10*, 5247–5250.
(9) For total synthesis of cortistatin A, see: (a) Shenvi, R. A.; Guerrero, C. A.; Shi, J.; Li, C.-C.; Baran, P. S. *J. Am. Chem. Soc.* **2008**, *130*, 7241–7243. (b) Nicolaou, K. C.; Sun, Y.-P.; Peng, X.-S.; Polet, D.; Chen, D. Y. K. *Angew. Chem., Int. Ed.* **2008**, *47*, 7310–7313. (c) Lee, H. M.; Nieto-Oberhuber, C.; Shair, M. D. *J. Am. Chem. Soc.* **2008**, *130*, 16864–16866. For approaches toward the cortistatin core, see: (d) Simmons, E. M.; Hardin, A. R.; Guo, X.; Sarpong, R. *Angew. Chem., Int. Ed.* **2008**, *47*, 6650–6653. (e) Dai, M.; Danishefsky, S. J. *Tetrahedron Lett.* **2008**, *49*, 6610–6612. (f) Craft, D. T.; Gung, B. W. *Tetrahedron Lett.* **2008**, *49*, 5931–5934. (g) Kürti, L.; Czakó, B.; Corey, E. J. *Org. Lett.* **2008**, *10*, 5247–5250.
(10) Overlay of stereochemical models was done using ChemBio3D Ultra 11.0 based on the minimal energy conformations of **1** and **12**.

Scheme 2. Synthesis of 7-Tributylstannyl Isoquinoline **20**



Scheme 3. Synthesis of **21** and **22**



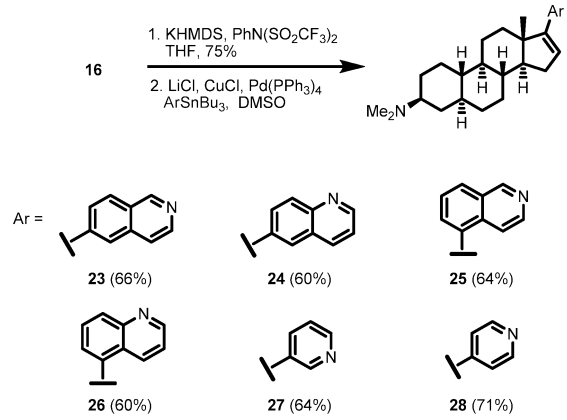
was coupled with 7-tributylstannyl isoquinoline (**20**) using the Corey/Han/Stoltz-modified Stille reaction to provide the desired analogue **12**.[12] 7-Tributylstannyl isoquinoline (**20**) was prepared by treatment of 7-bromoisoquinoline (**19**) with *n*-BuLi and tributyltin chloride (Scheme 2). 7-Bromoisoquinoline (**19**) was synthesized following a procedure described for 7-chloroisoquinoline,[13] affording a 1.5:1 mixture of 7-bromoisoquinoline and 5-bromoisoquinoline. Fortunately, the HBr salts of these isomers could be separated readily by crystallization. To arrive at α-dimethylamino ketone **13**, enone **17** was reduced to the corresponding β-alcohol using Li/NH_3/EtOH and subsequently converted to analogue **13** following a sequence similar to that described for **12**.

Detailed biological evaluation of **12** and **13** indicated that the 3β-dimethylamino diastereomer **12** is more active, and consequently further studies concentrated on compounds in the 3β-series. Intermediate **12** was used to prepare the related 17β-oriented isoquinoline **21** by diimide reduction of the double bond (potassium azodicarboxylate, AcOH)[14,15] and the tetrahydroisoquinoline derivative **22** by reductive methylation (CH_2O and $NaBH_3CN$), as shown in Scheme 3.

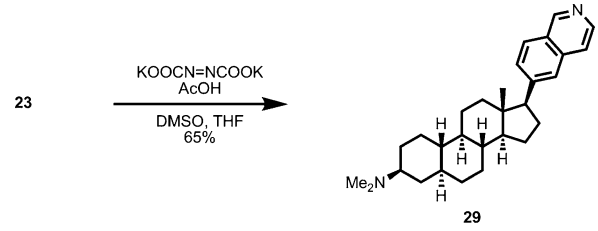
Intermediate **16** allowed systematic variation of the appendage at C17, as shown in Scheme 4. Specifically, compounds **23**−**28** were synthesized for biological studies from **16**. Further, the 17β analogue (**29**) of **23** was produced by diimide reduction (Scheme 5).

We also investigated a series of compounds in which the 3β-dimethylamino group was replaced by a 3β-pyrrolidino or 3β-morpholino group. These compounds were prepared as shown in Scheme 6.

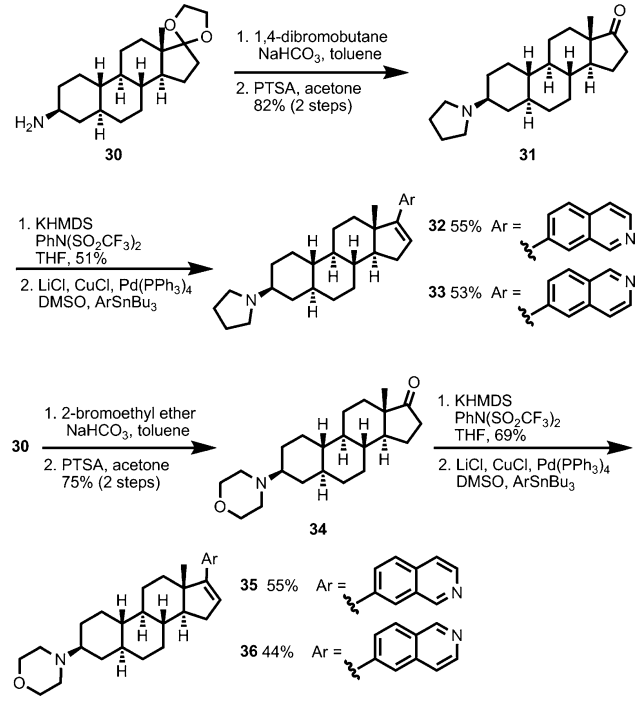
Scheme 4. Varying the Heterocycle at the C17 Position



Scheme 5. Reduction of **23**



Scheme 6. Preparation of Pyrrolidine and Morpholine Analogues of Compounds **12** and **23**



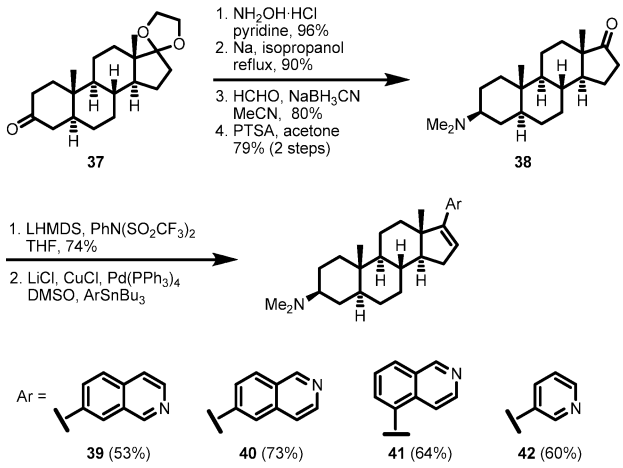
Finally, we prepared the steroidal analogues **39**−**42** based on the 19-norsteroids **12**, **23**, **25**, and **27**, as summarized in Scheme 7, for biological evaluation.[16]

Biological Evaluation of Cortistatin Analogues

The antiangiogenic activity of the new steroidal and 19-norsteroidal analogues of cortistatin were evaluated *in vitro* using assays for VEGF-stimulated HUVEC growth, migration, and tubular network formation. The data obtained indicates that

(11) Fedorova, O. I.; Pekarskaya, E. S.; Lukashina, I. V.; Grinenko, G. S. *Pharm. Chem. J.* **1974**, *8*, 462.
(12) Han, X.; Stoltz, B. M.; Corey, E. J. *J. Am. Chem. Soc.* **1999**, *121*, 7600–7605.
(13) We slightly modified Brown's procedure describing the preparation of 7-chloroisoquinoline: Brown, E. V. *J. Org. Chem.* **1977**, *42*, 3208–3209.
(14) Pasto, D. J.; Taylor, R. T. *Organic Reactions*; Wiley: Hoboken, NJ, 1991; Vol. 40, pp 91−150.
(15) Catalytic hydrogenation using Pd/C under a variety of conditions led to partial reduction of the isoquinoline. Raney nickel hydrogenation provided a very low yield of the isolated product.

Scheme 7. Synthesis of Analogues **39**−**42**



the most active of the compounds described above were **23**, **25**, and **27**. In the VEGF-stimulated migration of HUVECs, for instance, these compounds showed a 50% or greater inhibition vs controls at a concentration of 50 nM (Figure 3).

A. Cell Migration Studies. Cell migration is important for angiogenesis; therefore, it was examined whether the above-prepared compounds control HUVEC motility stimulated by VEGF using a transwell migration assay.[17] These cell migration assays revealed that compounds **23**, **25**, and **27** exhibited strong inhibitory activity at a concentration of 50 nM, while **24** and **26** were moderately active at this concentration and **28** was inactive (Figure 4). Lead compounds **12** and **13** exhibited moderate inhibitory activity at 200 nM concentration. Steroidal analogues **39**−**42**, based on the structure of **12** and **23**, were less active than the corresponding 19-norsteroidal analogues at 50 nM concentration. Reduced derivates of **12** (i.e., **21** and **22**) did not show inhibition at 50 nM concentration, but **21** was active at 200 nM concentration. Compound **29**, obtained by the reduction of **23**, was less active than the parent compound. Pyrrolidine and morpholine derivatives **32**, **33**, **35**, and **36** did not inhibit cell migration at 50 nM concentration. Although authentic samples of cortistatin A were not available for us to compare

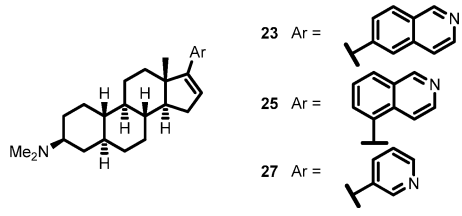


Figure 3. Most active analogues.

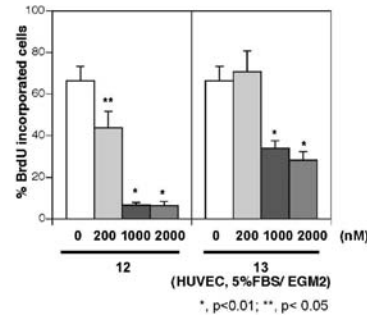


Figure 5. Inhibition of HUVEC growth by compounds **12** and **13**.

the activity of **1** and **12** directly, comparison of the literature data reported for **1** with the experimental results obtained for **12** suggests that the inhibitory effect of **12** on cell migration is similar to that of **1** and that compounds **23**, **25**, and **27** exhibit stronger inhibitory activity on VEGF-induced cell migration of HUVECs than **1**.[5] The IC_{50} of compound **23** for inhibition of VEGF-induced cell migration was 70.7 nM.

B. Studies of HUVEC Growth. The effect of the active compounds on HUVEC growth was examined by measuring nuclear incorporation of BrdU into DNA.[18] Lead compounds **12** and **13** inhibited BrdU incorporation at doses greater than 1 μM, respectively, and **12** inhibited BrdU incorporation at 200 nM concentration (Figure 5).

Compound **23**, which was the most active analogue based on migration assays, also exhibited strong inhibition of cell growth induced by VEGF, bFGF, and PDGF (platelet-derived growth factor), with IC_{50}'s of 16.7, 64.1, and 79.5 nM, respectively, when tested individually against each of these growth factors (see Figure 11, below) [IC_{50}(**1**) = 1.8 nM]. Compound **27** inhibited cell growth induced by VEGF at 50 nM concentration, but it inhibited growth induced by bFGF and PDGF only at higher concentrations (Figure 6).

Compounds **39**−**42** did not significantly inhibit cell growth induced by VEGF (20 ng/mL) at 200 nM, although they inhibited growth by about half at 1000 nM (Figure 7).

C. Studies of Tubular Network Formation. The effect of these compounds on *in vitro* angiogenesis was investigated using the Matrigel tube formation assay.[19,20] It was found that lead compounds **12** and **13** (2 μM) inhibited tubular network formation when cultured with VEGF in the basal EBM2 medium with a 0.5% serum, but **12** was more potent (Figure 8).

The most active compound based on cell migration and growth assays, **23**, produced significant inhibition of tubular network formation at 50 nM concentration when cultured with VEGF in the basal EBM2 medium (Figure 9). Quantified data

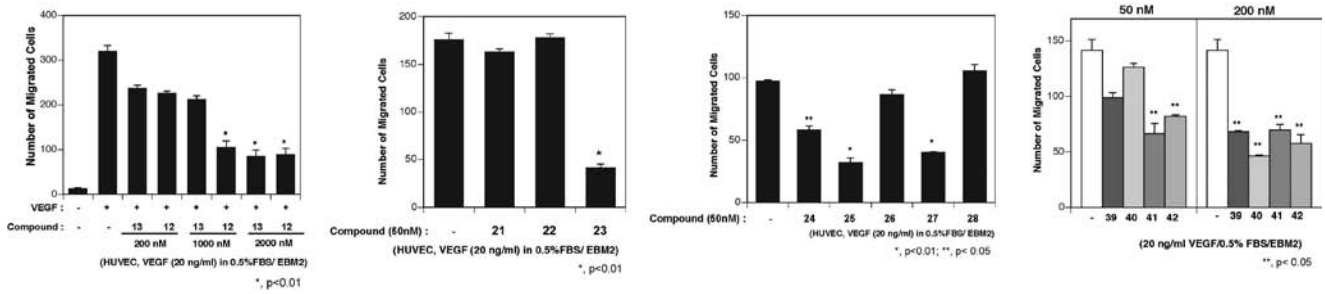


Figure 4. Inhibition of VEGF-induced migration of HUVECs by synthetic amino steroids and 19-norsteroids.

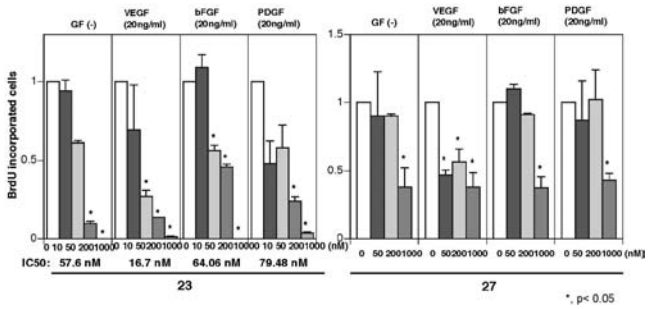


Figure 6. Inhibition of VEGF-, bFGF-, and PDGF-induced HUVEC growth by compounds **23** and **27**.

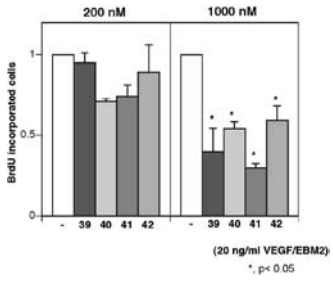


Figure 7. Inhibition of VEGF-induced HUVEC growth by compounds **39**−**42**.

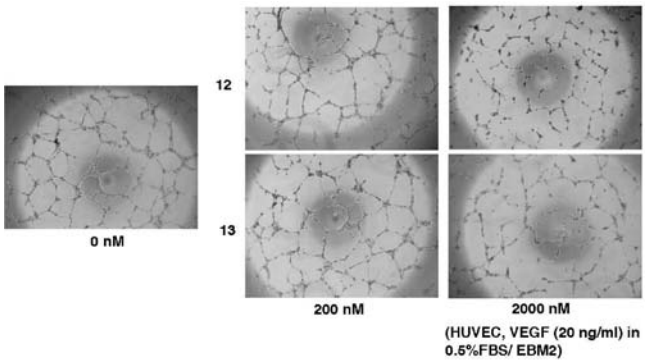


Figure 8. Inhibition of VEGF-induced tubular network formation by **12** and **13**.

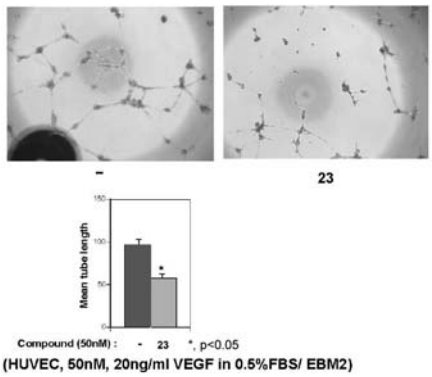


Figure 9. Inhibition of tubular network formation by **23**.

on the inhibition of VEGF-induced tubular network formation by cortistatin A (**1**) have not been reported.

D. *In Vivo* Studies with Mice Using Compounds 12 and 23. To begin exploring the clinical relevance of these compounds, the effect of compound **12** on retinal angiogenesis was examined by intravitreous injection of these compounds into the eyes of newborn mice.[21] Compound **12** appeared to inhibit retinal angiogenesis based on morphological analysis with 5−10 nmol in a single injection (Figure 10).

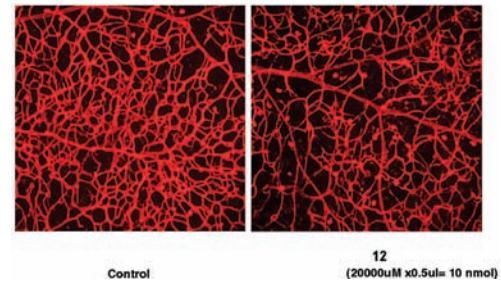


Figure 10. Effect of **12** on retinal vessel formation of P7 mice.

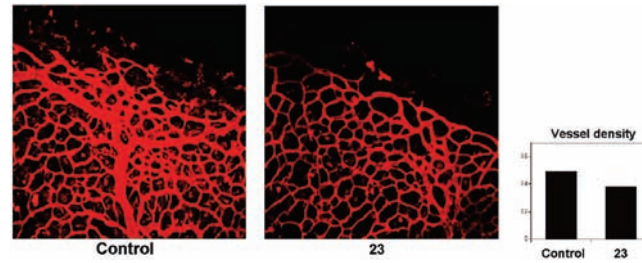


Figure 11. Effect of **23** on retinal vessel formation of P6 mice.

Based on the very impressive *in vitro* biological results obtained for compound **23**, inhibition of retinal vessel formation by **23** in P6 (6 days post birth) mice was examined. These experiments revealed that compound **23** produced significant inhibition of retinal angiogenesis in P6 mice after a single injection of 500 pmol of **23**. Compound **23** was more effective than lead compound **12**, which required more than 10 times higher doses (5−10 nmol) to produce similar effects (Figure 11). Similar *in vivo* experiments have not been reported for **1.** *In vitro* cell toxicity measurements of **12** and **23** with several cell cultures revealed no observable toxicity at 50 μM.

Conclusion

Based on the structure of the highly complex sterodial antiangiogenic natural products, the cortistatins, a series of analogues were designed and synthesized that inhibit capillary cell growth, migration, *in vitro* tube formation, and *in vivo* angiogenesis in the living retina. These compounds are structurally less complex and much more readily accessible by synthesis than the natural product. The most active and most promising of the new compounds reported herein were the 3β-dimethylamino-19-norsteroids with $\Delta^{16,17}$ unsaturation. The substituent at C17 could be varied somewhat, but a basic

(16) Reyes, M.; Rosado, A.; Alvarez, Y. M.; Ruiz, J. A.; Aguero, J.; Velez, H. *J. Chem. Res., Synop.* **2003**, 234–235.
(17) Shimizu, A.; Mammoto, A.; Italiano, J. E., Jr.; Pravda, E.; Dudley, A. C.; Ingber, D. E.; Klagsbrun, M. *J. Biol. Chem.* **2008**, *283*, 27230–27238.
(18) Huang, S.; Chen, C. S.; Ingber, D. E. *Mol. Biol. Cell* **1998**, *9*, 3179–3193.
(19) Grant, D. S.; Kinsella, J. L.; Kibbey, M. C.; LaFlamme, S.; Burbelo, P. D.; Goldstein, A. L.; Kleinman, H. K. *J. Cell Sci.* **1995**, *108*, 3685–3694.
(20) Mammoto, A.; Connor, K. M.; Mammoto, T.; Yung, C. W.; Huh, D.; Aderman, C. M.; Mostoslavsky, G.; Smith, L. E. H.; Ingber, D. E. *Nature (London, U.K.)* **2009**, *457*, 1103–1108.
(21) Chen, J.; Connor, K. M.; Aderman, C. M.; Smith, L. E. H. *J. Clin. Invest.* **2008**, *118*, 526–533.

heterocycle seemed important for bioactivity. These compounds inhibit the angiogenic effects of VEGF, and some have the ability to inhibit cell sensitivity to multiple angiogenic factors, the most active analogue being **23**, which exhibits highly potent antiangiogenic activity at low nanomolar concentrations in *in vitro* assays. Comparison of the biological activity of the synthetic compounds **23**, **25**, and **27** with published data on cortistatin A indicates that the synthetic compounds inhibit VEGF-induced cell migration of HUVECs more strongly than **1**. Most importantly, locally administered picomole quantities of **23** were shown to inhibit retinal vessel formation in P6 mice, a recognized animal model for ocular wet macular degeneration. These data indicate that these water-soluble, apparently nontoxic compounds (at 50 μM) may be suitable for local application to treat ocular wet macular degeneration, an important cause of blindness, as well as for treatment of various other angiogenesis-dependent diseases, including malignant and inflammatory conditions.

Acknowledgment. L.K. is a Fellow of the Damon Runyon Cancer Research Foundation (DRG: 1961-07). This work was supported by NIH grants CA45548 and CA55833 (to D.E.I.). D.E.I. is a recipient of a Department of Defense Breast Cancer Innovator Award.

Supporting Information Available: Detailed experimental procedures and characterization data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

JA902601E