Synthesis and Evaluation of a Novel Nonsteroidal-Specific Endothelial Cell Proliferation Inhibitor

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Abstract: The identification of agents with specific antiproliferative or cytostatic activity against endothelial cells has significant value for the treatment of pathologies associated with angiogenesis, including solid tumors. Here, we describe a novel substituted dibenzo[*b,d*]pyran-6-one scaffold, exemplified by structures **9a** and **10**, and report preliminary in vitro activity data indicating that this scaffold is a promising lead for the development of specific inhibitors of endothelial cell proliferation.

Introduction. Angiogenesis, the growth of new blood vessels, involves the proliferation of endothelial cells in response to specific growth stimuli such as vascular endothelial growth factor (VEGF) of basic fibroblast growth factor (bFGF). The growth and maintenance of solid tumors is highly dependent on neovascularization and can be regulated by compounds that interfere with either the stimulation or proliferation of endothelial cells.[1] Consequently, the control of angiogenesis continues to be an attractive area for the development of novel therapeutic agents.[2]

One such agent is 2-methoxyestradiol (2-ME_2, Panzem), an endogenous metabolite of estradiol (E_2) that has been demonstrated to inhibit both tumor growth and angiogenesis in vivo.[3,4,7−13] However, in addition to its antiangiogenic activity, 2-ME_2 induces apoptosis in actively proliferating cells and exhibits general nonspecific antiproliferative effects against a wide range of human cancer cell cultures. It also has limited oral bioavailability in mice[14] and is rapidly cleared following administration.

In light of these potential pharmacological and therapeutic limitations, there remains an ongoing interest in the identification of more potent and bioavailable nonsteroidal analogues of 2-ME_2 with specific inhibitory activities against proliferating endothelial cells. Hence, in the course of our drug discovery programs, we were intrigued by the structural similarities between a series of substituted dibenzo[*b,d*]pyran-6-ones and 2-ME_2 and were curious to determine whether they had selective antiangiogenic activity. Here, we report the synthesis of substituted dibenzo[*b,d*]pyran-6-ones along with preliminary in vitro biological activity that demonstrates that they are specific inhibitors of proliferating endothelial cells.

Synthesis. There are a number of methods that have been utilized to prepare dibenzo[*b,d*]pyran-6-ones.[15−17] Our initial efforts with these methods were unsuccessful, although we did not explore them exhaustively. We subsequently turned our attention to the report where a NO_2 containing dibenzo[*b,d*]pyran-6-one was synthesized.[18] In this case, Suzuki coupling of an arylboronic acid with the electron-withdrawing NO_2 containing bromobenzene generated the requisite biphenyl that cyclized efficiently to the lactone.

Since methyl 5-acetylsalicylate was commercially available, we modified the procedure slightly and the route to the dibenzo[*b,d*]pyran-6-ones **9a** and **10** is given in Scheme 1. Both compounds still relied on Suzuki coupling but now with a common aryl triflate and an arylboronic acid. The requisite arylboronic acids were either commercially available (**5a**) or prepared as shown in Scheme 1 (**5b**). 2-Bromo-5-hydroxy-4-methoxybenzaldehyde was treated with K_2CO_3 and benzyl bromide in acetonitrile to give the benzyl-protected aryl aldehyde **2** that was then oxidized with mCPBA in CH_2Cl_2 to the phenol **3**. To set up for the future lactone formation, **3** was treated with NaH and CH_3I in anhydrous THF to give the aryl methyl ether **4**. Then, by use of metal halogen exchange conditions, **4** was treated with nBuLi in dry THF at −78 °C followed by triisopropyl borate, which gave the boronic acid **5b**. The other partner for the Suzuki coupling reactions was prepared by making the triflate **6** from methyl 5-acetylsalicylate using triflic anhydride in CH_2Cl_2 containing pyridine at 0 °C. Suzuki coupling of the arylboronic acids **5a**,**b** with **6** using Na_2CO_3 and a catalytic amount of $Pd(PPh_3)_4$ in absolute ethanol and DME yielded the biphenyl esters **7a**,**b**. Saponification with KOH gave the free acids **8a**,**b** that upon reaction with thionyl chloride and $AlCl_3$ gave the dibenzo[*b,d*]pyran-6-ones **9a**,**b**. We have since determined that $AlCl_3$ is not required for lactone formation. The 3-hydroxy dibenzo[*b,d*]pyran-6-one **10** was obtained by in situ catalytic hydrogenation of **9b** with NH_4HCO_2 and 10% Pd/C in absolute ethanol and ethyl acetate. The yield of this debenzylation reaction was very low (27%), and we subsequently improved the yield to greater than 80% and will be reporting the methodology elsewhere. The structures of the intermediates and final compounds were confirmed by ^1H NMR with elemental analysis determined on the final compounds.

Although the dibenzo[*b,d*]pyran-6-one scaffold has not been used frequently in pharmaceuticals, it is found in naturally occurring compounds exemplified by alternariol,[15] ellagic acid,[15] and the benzo[*d*]naphthopyran-6-one gilvocarcin V[19] as shown in Scheme 2. Ellagic acid inhibits the mutagenicity of polycyclic aromatic hydrocarbons in vivo, and gilvocarcin V has significant in vitro antitumor activity when activated by low-energy light.

In Vitro Biology. The antiproliferative effects of **9a** and **10** compared to 2-ME_2 were evaluated using primary human umbilical vein endothelial cells (HUVECs) in the presence of bFGF[20] as shown in Figure 1.

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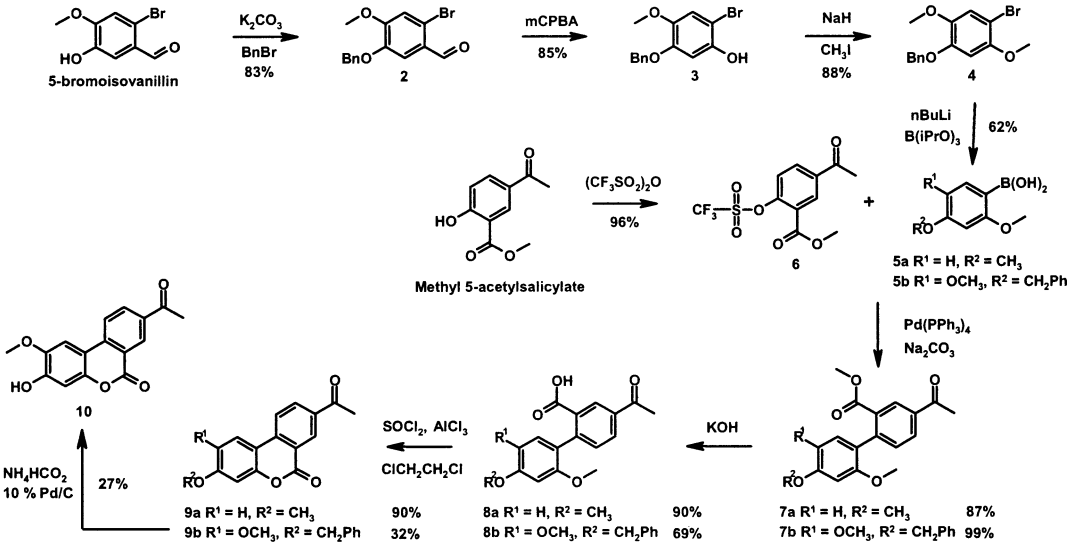
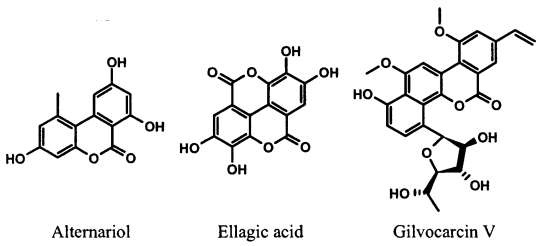
Scheme 1



Scheme 2



Both **9a** and **10** caused dose-dependent inhibition of bFGF-stimulated endothelial cell proliferation. The activity of these compounds was comparable to that of 2-ME_2. At a concentration of 10 μM, 2-ME_2 reduced cell counts below the level of the untreated controls, indicating a possible cytotoxic effect. In contrast, **9a** and **10** did not reduce cell counts below the control level, suggesting that they may be cytostatic rather than cytotoxic agents over this concentration range.

Under the same treatment concentrations, neither **9a** nor **10** significantly inhibited or stimulated the proliferation of MDA-MB-231 human breast cancer cells, estradiol-responsive MCF-7 human breast cancer cells, or HCT-116 human colorectal carcinoma cells.[21] In the same assays, 2-ME_2 was significantly antiproliferative. These observations suggest that the inhibitory effects of **9a** and **10** are more specific than those of 2-ME_2. The ability to selectively inhibit endothelial cell proliferation has important therapeutic implications, especially for the treatment of nononcological diseases and the use of these compounds in combination therapy.

In light of the structural similarities between **9a** and **10** with estradiol (E_2), it was important to examine the ability of these compounds to bind with the estrogen receptor (ER). In a competitive binding assay, neither **9a** nor **10** significantly inhibited the binding of 0.5 nM radiolabeled E_2 with either ER_α (Figure 2, left panel) or ER_β (Figure 2, right panel) at 10 μM. At the same concentration, the positive control DES (diethylstilbesterol) inhibited E_2 binding by >75%. At 10 μM, 2-ME_2 inhibited E_2 binding to ER_α by 60% and to ER_β by 25%. Measurable binding of 2-ME_2 at this high dose is consistent with previously reported values.[22] The

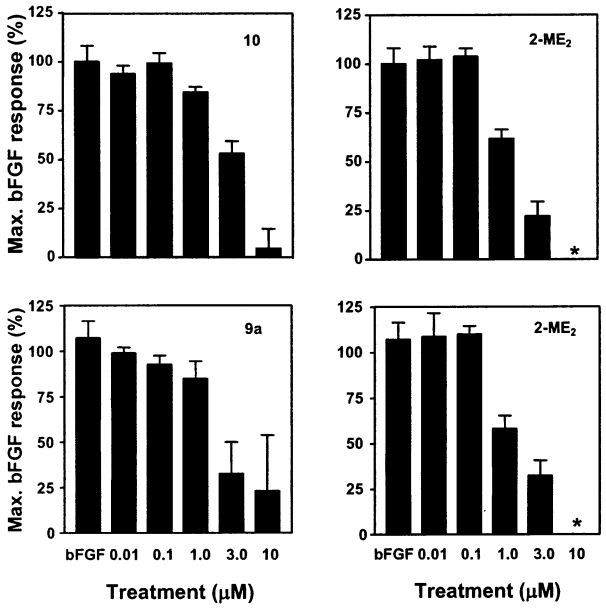


Figure 1. Inhibition of the proliferation of human endothelial cells by **9a**, **10**, and 2-Me_2. Top panel: **10** vs 2-ME_2 in the HUVEC proliferation assay. The cells were incubated with bFGF alone or in the presence of increasing amounts of **10** or 2-ME_2 as indicated. The maximum growth factor response was set to 100%, and the results are expressed as the percentage of cells remaining compared to the maximal response. The amount of cells remaining in the wells that were not treated with bFGF (starved) was set to 0 (not shown). The asterisk (*) indicates that the amount of cells remaining was below that of the unstimulated cells. The data represent the mean ± SEM (n = 11). Bottom panel: as in the top except **9a** is compared to 2-ME_2 (n = 5).

observations that **9a** and **10** do not compete with E_2 for the estrogen receptor and do not have a stimulatory effect on E_2-responsive MCF-7 breast cancer cells provide strong evidence that these compounds are unlikely to have estrogenic liabilities such as uterotrophic activity.

Computational Mechanism of Action Studies. The mechanism of action of 2-ME_2 is not well understood, and the molecular target remains unknown. Several hypotheses have been proposed including the

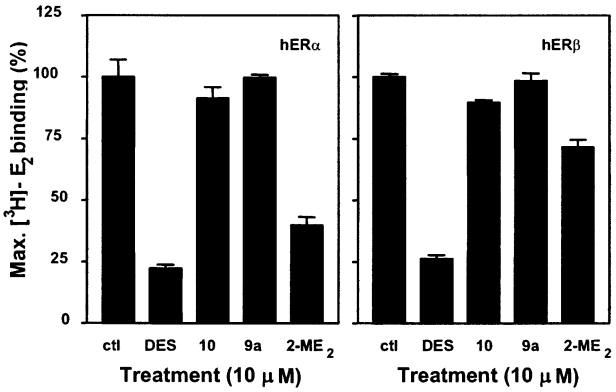


Figure 2. Effect of **9a**, **10**, and 2-ME_2 with the human estrogen receptors. In vitro transcribed−translated ER_α and ER_β were incubated with 0.5 nM [^{3}H]E_2 in the presence of 10 μM diethylstilbestrol (DES), **9a**, **10**, or 2-ME_2. The maximum amount of specifically bound radioligand was determined in the presence of vehicle and expressed as 100% (control). The data represent the mean ± SEM (n = 3).

inhibition of tubulin polymerization,[23] the up-regulation of p53,[24,25] the induction of caspases,[26] and the inhibition of superoxide dismutase activity.[27] In each of these proposed mechanisms, treatment of cells with 2-ME_2 ultimately leads to either cell arrest or apoptosis by interfering with the regulation of the cell cycle.[28] Despite its low affinity to E_2, a recent report has documented that the action of 2-ME_2 is independent of the ERs.[22] Similarly, we have ruled out ERs as potential targets for the dibenzo[*b*,*d*]pyran-6-ones because we have observed no significant interaction between ER_α and ER_β with **9a** and **10**.

Although we have only just begun to address the issue of a mechanism of action, preliminary studies indicate that they do not induce the expression of caspases,[29] suggesting that they may inhibit endothelial cell proliferation by a mechanism other than apoptosis. It was also determined that the effect of **9a** was not dependent on p53.[21]

On the basis of flexible overlays of 2-ME_2 and several colchicine analogues, it appears unlikely that 2-ME_2 would inhibit tubulin polymerization by binding to the colchicine site,[30] a mechanism that has been invoked for 2-ME_2.[23] For several other potential targets, we performed pairwise flexible molecular alignments[31] of 2-ME_2/**9a**/**10** with low nanomolar inhibitors of these targets to evaluate the likelihood of any of the three molecules competing for the corresponding binding site. In all cases 1000 overlays were carried out using a Cartesian perturbation of 0.5 Å, with all other conditions being equal to those previously described.[31] On the basis of alignment with indanocene, PD153035, and SU5416, it appears unlikely that **9a** and **10** share the binding site of these molecules on microtubules, the EGFR-TK receptor, and the VEGF2 (Flk-1/KDR) receptor, respectively. In a similar manner, flexible alignments of **9a** and **10** with colchicine analogues confirm that the colchicine site is an unlikely target for these molecules.

Flexibly docking **9a** and **10**, using the GOLD software,[32] to the site where a known low nanomolar quinolinone type inhibitor of this receptor binds[33] to the 1VR2 crystal structure[34] also suggests that it is unlikely that VEGF2 (KDR) is responsible for the mode of action. Further studies are underway in an attempt to identify the putative molecular target for these molecules.

We also thought it might be the hydrolyzed lactones that may be responsible for the biological activity; however, the precursor esters **7a**,**b** and acids **8a**,**b** were inactive in the HUVEC assay.

Conclusions. In view of its oral in vivo efficacy against solid tumors, low toxicity, and lack of estrogenicity,[35] it has been argued that the natural metabolite 2-ME_2 is close to being an ideal antiproliferative agent. However, low bioavailability in mice,[14] rapid clearance, and lack of specificity for endothelial cells[35] may limit the potential therapeutic applications of 2-ME_2. The acetylated dibenzo[*b*,*d*]pyran-6-ones **9a** and **10** appear to provide an opportunity to improve upon the therapeutic profile of 2-ME_2. These compounds are nearly as effective inhibitors of stimulated HUVEC proliferation as 2-ME_2. They are also unlikely to be estrogenic. At the same time, the nonsteroidal scaffolds of **9a** and **10** open a route for the identification of analogues that are not subject to the same well-established clearance mechanisms as 2-ME_2. More importantly, our in vitro studies indicate that the antiproliferative activity of **9a** and **10** is more selective for endothelial cells than that of 2-ME_2.

This finding is of practical significance for the management of diseases in which angiogenesis and neovascularization are the primary or exclusive targets of treatment, e.g., retinopathies and inflammatory conditions. In oncological therapy, compounds that act specifically against endothelial cells are likely to have the greatest clinical value when administered in combination with other cytotoxic agents. By exploitation of independent mechanisms of action in distinct cell types, it may be possible to mitigate against the rapid onset of resistance to more general antimitotic treatments. The discovery that the dibenzo[*b*,*d*]pyran-6-ones are specific inhibitors of stimulated endothelial cells and not generally active against proliferating cells is a significant step in the development of such a therapeutic approach.

We will be reporting the results of in vivo efficacy studies separately.[21] In addition, since with steroids, clearance and metabolism via phenol conjugation and demethylation are well-established mechanisms, we will be evaluating these pathways for **9a** and **10** as well. Also, further research is in progress to identify the mode of action and molecular target of these compounds.

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

References

(1) Fantl, W. J.; Rosenberg, S. In *Annual Reports in Medicinal Chemistry*; Doherty, A. M., Ed.; Academic Press: San Diego, CA, 2000; Vol. 35, pp 123−133.
(2) Matter, A. Tumor angiogenesis as a therapeutic target. *Drug Discovery Today* **2001**, *6*, 1005−1024.
(3) Fotsis, T.; Zhang, Y.; Pepper, M. S.; Adlercreutz, H.; Montesano, R.; Nawroth, P. P.; Schweigerer, L. The endogenous oestrogen metabolite 2-methoxyestradiol inhibits angiogenesis and suppresses tumor growth. *Nature* **1994**, *368*, 237−239.

(4) Folkman, J.; Watson, K.; Ingber, D. E.; Hanahan, D. Induction of angiogenesis during the transition from hyperplasia to neoplasia. *Nature* **1989**, *339*, 58−61.
(5) Schumacher, G.; Kataoka, M.; Roth, J. A.; Mukhopadhyay, T. Potent antitumor activity of 2-methoxyestradiol in human pancreatic cancer cell lines. *Clin. Cancer Res.* **1999**, *5*, 493−499.
(6) Wassberg, E. Angiostatic treatment of neuroblastoma. *Upsala J. Med. Sci.* **1999**, *104*, 1−24.
(7) Blood, C. H.; Zetter, B. R. Tumor interaction with the vasculature: Angiogenesis and tumor metastasis. *Biochim. Biophys. Acta* **1990**, *1032*, 89−118.
(8) Klauber, N.; Parangi, S.; Flynn, E.; Hamel, E.; D'Amato, R. J. Inhibition of angiogenesis and breast cancer in mice by the microtubule inhibitors 2-methoxyestradiol and taxol. *Cancer Res.* **1997**, *57*, 81−86.
(9) Yue, T. L.; Wang, X. K.; Louden, C. S.; Gupta, S.; Pillarisetti, K.; Gu, J. L.; Hart, T. K.; Lysko, P. G.; Feuerstein, G. Z. 2-Methoxyestradiol, an endogenous metabolite, induces apoptosis in endothelial cells and inhibits angiogenesis: Possible role for stress-activated protein kinase signaling pathway and fas expression. *Mol. Pharmacol.* **1997**, *51*, 951−962.
(10) Tsukamoto, A.; Kaneko, Y.; Toshida, T.; Han, K.; Ichinose, M.; Kimura, S. 2-Methoxyestradiol, an endogenous metabolite of estrogen enhances apoptosis and β-galactosidase expression in vascular endothelial cells. *Biochem. Biophys. Res. Commun.* **1998**, *248*, 9−12.
(11) Josefsson, E.; Tarkowski, A. Suppression of type II collagen-induced arthritis by the endogenous estrogen metabolite 2-methoxyestradiol. *Arthritis Rheum.* **1997**, *40*, 154−163.
(12) Reiser, F.; Way, D.; Bernas, M.; Witte, M.; Witte, C. Inhibition of normal and experimental endothelial cell proliferation and cell cycle progression by 2-methoxyestradiol. *Proc. Soc. Exp. Biol. Med.* **1998**, *219*, 211−216.
(13) *2-Methoxyestradiol: A Novel Endogenous and Anti-Angiogenic Agent;* Pribula, V. S., LaVallee, T. M., Green, S. J., Eds.; Humana Press, Inc.: Totowa, NJ, 2002.
(14) Squillace, D. P.; Reid, J. M.; Kuffel, M. J.; Ames, M. M. Bioavailability and *in vivo* metabolism of 2-methoxyestradiol in mice. *Proc. Am. Assoc. Cancer Res.* **1998**, *39*, 523.
(15) Alo, B. I.; Kandil, A.; Patil, P. A.; Sharp, M. J.; Siddiqui, M. A.; Snieckus, V. Sequential directed ortho metalation−boronic acid cross-coupling reactions. A general regiospecific route to oxygenated dibenzo[*b*,*d*]pyran-6-ones related to ellagic acid. *J. Org. Chem.* **1991**, *56*, 3763.
(16) Langer, P.; Saleh, N. N. R.; Freifeld, I. Efficient synthesis of biaryl lactones by domino retro-Michael-aldol lactonization reactions. *Chem. Commun.* **2002**, *2*, 168−169.
(17) Weidner-Wells, M. A.; Altom, J.; Fernandez, J.; Fraga-Spano, S. A.; Hilliard, J.; Ohemeng, K.; Barret, J. F. DNA gyrase inhibitory activity of ellagic acid derivatives. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 97−100.
(18) Edwards, J. P.; West, S. J.; Marschke, K. B.; Mais, D. E.; Gottardis, M. M.; Jones, T. K. 5-Aryl-1,2-dihydro-5*H*-chromeno-[3,4-*f*]quinolines as potent, orally active, non-steroidal progesterone receptor antagonists. The effect of D-ring substituents. *J. Med. Chem.* **1998**, *41*, 303.
(19) McGee, L. R.; Confalone, P. N. On the photobiology of the Gilvocarcins. Total synthesis of Defucogilvocarcin V and a related photoactive vinyl phenol. *J. Org. Chem.* **1988**, *53*, 3695.
(20) Adapted from the following. Teicher, B. A.; Alvarez, E.; Menon, K.; Esterman, M. A.; Considine, E.; Shih, C.; Faul, M. M. Antiangiogenic effects of a protein kinase Cbeta-selective small molecule. *Cancer Chemother. Pharmacol.* **2002**, *49*, 69−77.
(21) Tremblay, G. B.; Pagé, M.; Peter, M.; Feher, M.; Dunn-Dufault, R.; Redden, P.; Mercure, J.; Schmidt, J. M. Unpublished results.
(22) LaVallee, T. M.; Zhan, X. H.; Herbstritt, C. J.; Kough, E. C.; Green, S. J.; Pribluda, V. S. 2-Methoxyestradiol inhibits proliferation and induces apoptosis independently of estrogen receptors alpha and beta. *Cancer Res.* **2002**, *62*, 3691−3697.
(23) D'Amato, R. J.; Lin, C. M.; Flynn, E.; Folkman, J.; Hamel, E. 2-Methoxyestradiol, an endogenous mammalian metabolite, inhibits tubulin polymerization by interacting at the colchicine site. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 3964−3968.
(24) Mukhopadhyay, T.; Roth, J. A. Induction of apoptosis in human lung cancer cells after wild-type p53 activation by methoxyestradiol. *Oncogene* **1997**, *14*, 379−384.
(25) Seegers, J. C.; Lottering, M. L.; Grobler, C. J.; van Papendorp, D. H.; Habbersett, R. C.; Shou, Y.; Lehnert, B. E. The mammalian metabolite, 2-methoxyestradiol, affects p53 levels and apoptosis induction in transformed cells but not in normal cells. *J. Steroid Biochem. Mol. Biol.* **1997**, *62*, 253−267.
(26) Lin, H. L.; Liu, T. Y.; Wu, C. W.; Chi, C. W. 2-Methoxyestradiol-induced caspase-3 activation and apoptosis occurs through G(2)/M arrest dependent and independent pathways in gastric carcinoma cells. *Cancer* **2001**, *92*, 500−509.
(27) Huang, P.; Feng, L.; Oldman, E. A.; Keating, M. J.; Plunkett, W. Superoxide dismutase as a target for the selective killing of cancer cells. Nature **2000**, *407*, 390−395.
(28) Pribluba, V. S.; Gubish, E. R., Jr.; LaVallee, T. M.; Treston, A.; Swartz, G. M.; Green, S. J. 2-Methoxyestradiol: an endogenous antiangiogenic and antiproliferative drug candidate. *Cancer Metastatis Rev.* **2000**, *19*, 173−179.
(29) Tremblay, G. B.; Pagé, M. Unpublished results.
(30) Feher, M.; Schmidt, J. M. Multiple flexible alignment with SEAL: a study of molecules acting on the colchicine binding site. *J. Chem. Inf. Comput. Sci.* **2000**, *40*, 495−502.
(31) Feher, M.; Schmidt, J. M. Identifying potential binding modes and explaining partitioning behavior using flexible alignments and multidimensional scaling. *J. Comput.-Aided Mol. Des.* **2001**, *15*, 1065−1083.
(32) Jones, G.; Wilett, P.; Glen, R. C.; Leach, A. R.; Taylor, R. Development and validation of a genetic algorithm for flexible docking. *J. Mol. Biol.* **1997**, *267*, 727−748.
(33) Fraley, M. E.; Antanavage, J.; Arrington, K. L.; Ciecko, P. A.; Coll, K. E.; Gibbs, J. B.; Hambaugh, S. R.; Hartman, G. D.; Heimbrook, D. C.; Hoffman, W. F.; Hungate, R. W.; Kohl, N. E.; Mao, X.; McFall, R. C.; Rands, E.; Rickert, K.; Sepp-Lorenzino, L.; Shipman, J. M.; Tebben, A. J.; Thomas, K. A. Discovery, synthesis, SAR and in vitro/in vivo characterization of a novel quinolinone class of VEGFR-2 (KDR) kinase inhibitors. *Abstracts of Papers*. 224th National Meeting of the American Chemical Society, Boston, MA, 2002; American Chemical Society: Washington, DC, 2003; MEDI 221.
(34) Berman, H. M.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T. N.; Weissig, H.; Shindyalov, I. N.; Bourne, P. E. The Protein Data Bank. *Nucleic Acids Res.* **2000**, *28*, 235−242.
(35) Pribluda, V. S.; LaVallee, T. M.; Green, S. J. In *The New Angiotherapy*; Fan, T.-P. D., Kohn, E. C., Eds.; Humana Press: Totowa, NJ, 2002; pp 387−407.

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