Evaluation of the Potential Cancer Chemotherapeutic Efficacy of Natural Product Isolates Employing in Vivo Hollow Fiber Tests[1]

Qiuwen Mi,[†] Daniel Lantvit,[†] Eulenia Reyes-Lim,[†] Heebyung Chai,[†] Weimin Zhao,[†,‡] Ik-Soo Lee,[†,§] Sergio Peraza-Sánchez,[†,⊥] Olipa Ngassapa,[†,∥] Leonardus B. S. Kardono,[▽] Soedarsono Riswan,[○] Melinda G. Hollingshead,[⊗] Joseph G. Mayo,[⊗] Norman R. Farnsworth,[†] Geoffrey A. Cordell,[†] A. Douglas Kinghorn,[†] and John M. Pezzuto*[,†]

Program for Collaborative Research in the Pharmaceutical Sciences, and Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, Chicago, Illinois 60612, and Biological Testing Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute−Frederick Cancer Research and Development Center, Frederick, Maryland 21701

Received June 29, 2001

The hollow fiber test has been developed for the preliminary in vivo assessment of cancer chemotherapeutic efficacy of selected natural products. Using this model, we have established growth conditions for HL-60, HUVEC, Ishikawa, KB, KB-V1, LNCaP, Lu1, MCF-7, Mel2, P-388, and SW626 cells implanted at the intraperitoneal (i.p.) and subcutaneous (s.c.) compartments of athymic mice. Five cytotoxic natural product isolates (**2**−**6**) were tested in this model, along with paclitaxel (taxol) (**1**). Among the compounds tested, dioscin (**2**) and 13-methoxy-15-oxozoapatlin (**3**) were found to be active, indicating their potential to function as cancer chemotherapeutic agents. On the other hand, ochraceolide A (**4**), α-lapachone (**5**), and 2-(1-hydroxyethyl)naphtha[2,3-*b*]furan-4,9-quinone (**6**), all of which were significantly cytotoxic to cultured mammalian cells, did not mediate significant responses with the hollow fiber model. In further xenograft studies using KB cells implanted at the subcutaneous site, compound **3** mediated a statistically significant response which was consistent with the response observed at the subcutaneous compartment in the hollow fiber tests. In sum, these studies illustrate the usefulness of the hollow fiber model in natural product drug discovery programs. Preliminary indications of potential therapeutic efficacy can be provided quickly at relatively low expense. Agents capable of mediating a response at the subcutaneous site would appear to warrant greatest attention.

The National Cooperative Natural Product Drug Discovery Group (NCNPDDG) program of the U.S. National Cancer Institute is one of the most extensive and well-organized efforts for the discovery of natural product cancer chemotherapeutic agents.[1] In our consortial group project, terrestrial plants have been used for investigation,[2] and one of the primary procedures of selection has involved analyses of dose−response patterns observed with panels of cultured human cancer cell lines.[3] The resulting dose−response patterns have proven useful for selecting starting materials for the isolation of active compounds. However, advancement of potential anticancer agents from discovery using in vitro screens to preclinical development requires the demonstration of efficacy in one or more animal models of neoplastic disease. Most such models require considerable quantities of test compound, as well as the substantial commitment of time and resources. Since therapeutic leads that appear promising with in vitro models are often less effective or not active against solid tumors, it would be desirable to follow an experimental paradigm that should help to focus on leads with the greatest promise of actually mediating a therapeutic response. One method for providing a preliminary indication of therapeutic efficacy has been described by Hollingshead et al.[4−7]

The majority of human tumor cell lines currently employed in cell culture can be grown inside hollow fibers[4,5] to form a heterogeneous solid tumor model. These semipermeable hollow fibers containing human tumor cells are implanted at the intraperitoneal or subcutaneous compartments of host mice, and the mice are treated with test substances of interest. Through determination of the potential to inhibit cell growth versus potential to mediate a toxic response toward the host, a preliminary estimate of therapeutic efficacy is provided in a cost- and time-effective manner.[5] The hollow fiber model does not fully mimic classical murine xenograft systems, where a range of complex interactions and phenomena occur when human tumor cells are growing in and interacting with the host. However, based on data generated with hollow fiber tests, conventional in vivo drug development resources can be focused on those compounds that have the greatest promise for use as chemotherapeutic agents.

In previous work, we have employed the BC1 (human breast carcinoma), Col2 (human colon carcinoma), KB (human oral epidermoid carcinoma), KB-V1 (vinblastine-resistant KB cells), LNCaP (human prostate carcinoma), Lu1 (human lung carcinoma), MCF-7 (human breast carcinoma), Mel2 (human melanoma), P-388 (mouse lymphoid neoplasm), SW626 (human ovarian adenocarcinoma), HL-60 (human promyelocytic leukemia), HUVEC (human umbilical vein endothelial), and Ishikawa (human endometrial carcinoma) cell lines to assess the growth inhibitory

* To whom correspondence should be addressed. Tel: (312) 996-5967. Fax: (312) 996-2815. E-mail: jpezzuto@uic.edu.
[†] University of Illinois at Chicago.
[‡] Present address: Department of Phytochemistry, Shanghai Institute of Materia Media, Chinese Academy of Sciences, Shanghai, 200031, People's Republic of China.
[§] Present address: College of Pharmacy, Chonnam National University, Kwangju 500-757, Korea.
[⊥] Present address: Centro de Investigacion Cientifica de Yucatan, Merida, Yucatan, Mexico 97200.
[∥] Present address: Faculty of Pharmacy, Mihimbili University College of Health Sciences, P.O. Box 65013, Dar es Salaam, Tanzania.
[▽] Research and Development Chemistry, Indonesian Institute of Science, Serpong, 15310 Tangerang, Indonesia.
[○] Herbarium Bogoriense, Research and Development Center for Biology, Indonesian Institute of Science, 16122 Bogor, Indonesia.
[⊗] National Cancer Institute-Frederick Cancer Research and Development Center.

10.1021/np010322w CCC: $22.00
Published on Web 05/14/2002

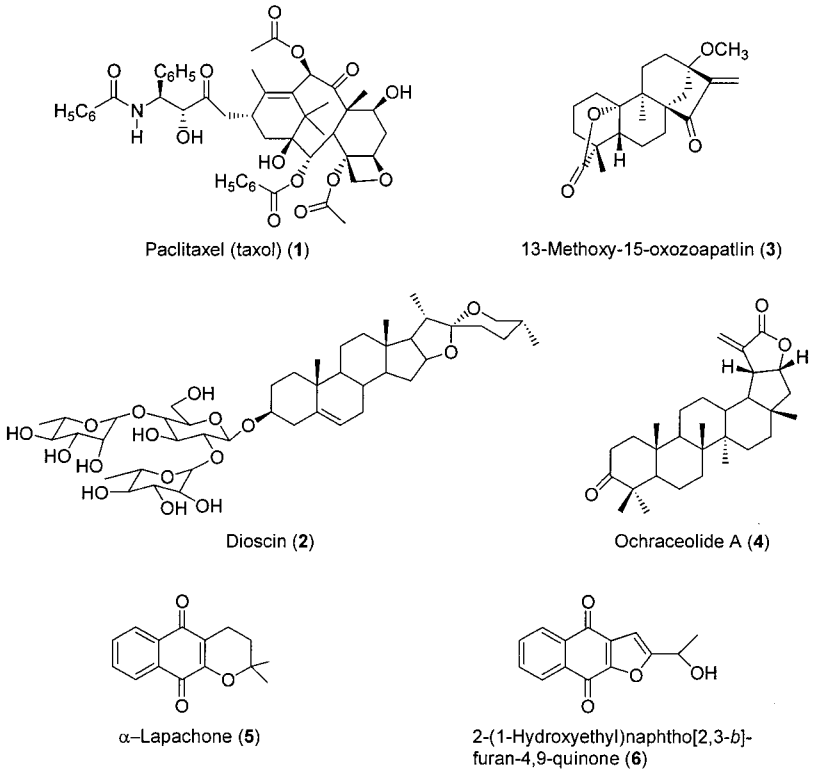

Figure 1. Chemical structures of **1**−**6**.

Table 1. Cytotoxic Activity of Compounds **1**−**6** with Cultured Cells[a]

compound	BC1	Col2	KB	KB-V1	LNCaP	Lu1	MCF-7	Mel2	P-388	SW626
1	0.02	0.02	0.02	>2.3	0.02	0.01	0.02	0.07	0.02	0.00001
2	3.8	2.8	20.7	>23	1.7	1.2	22.4	1.8	21.9	3.1
3	3.5	3.8	6.4	5.2	1.2	2.0	0.9	1.2	0.3	0.6
4	32.3	19.2	20.4	>44.2	13.1	24.3	16.6	14.8	3.5	35.4
5	27.7	40.9	53.7	34.3	29.3	21.9	38.0	7.9	0.8	12.4
6	13.2	7.4	2.1	4.9	14.5	0.8	2.5	1.2	0.4	1.2

[a] Results are expressed as IC_{50} values (concentration required to inhibit cell growth by 50%) in μM. Data represent the means of the two independent experiments, with each concentration tested in triplicate. Assays were performed as described in the Experimental Section.

potential of thousands of extracts derived from plant materials.[2,3] We currently report the growth characteristics and susceptibilities of these cells in the hollow fiber model. A number of pure isolates previously examined with mammalian cells in culture were evaluated with the in vivo hollow fiber model, and the compounds showing greatest promise were further investigated with classical xenograft models. The standard chemotherapeutic agent, paclitaxel (**1**), formerly known in the scientific literature as taxol, was used as a positive control. These studies illustrate the potential utility of in vivo hollow fiber testing as a component of natural product cancer chemotherapeutic drug discovery programs.

Results and Discussion

During the course of our studies directed toward the discovery of natural product cancer chemotherapeutic agents,[2] a variety of active molecules have been characterized. Some examples are compounds **2**−**6**, the structures of which are illustrated in Figure 1. Also, as summarized in Table 1, with Lu1, SW626, LNCaP, Mel2, BC1, and Col2 cells, **2** demonstrated IC_{50} (50% inhibitory concentration) values in the range 1.2−3.8 μM. Marginal activities were observed with KB, MCF-7, and P-388 cells, and the growth of KB-V1 cells was not affected. Compounds **3**, **5**, and **6** mediated growth inhibitory effects with all tested cell lines, and **4** was moderately active with all cell lines, except KB-V1. All cell lines were highly susceptible to **1**, with the exception of KB-V1.

To further assess the biological potential of these isolates, we tested their efficacy with the in vivo hollow fiber model. The hollow fiber studies were designed to determine if pharmacologically active concentrations of test compounds could reach tumor cells growing in two distinct physiologic compartments. Differences in activity against cells contained in the i.p. and s.c. fibers can aid in assessing the effect of hepatic pass through, thus providing information for dose estimations and administration routes for more extensive in vivo testing.[5] As a first step, preliminary evaluations were performed with each cell line by preparing hollow fiber samples with three cell densities (5×10^5, 10^6, and 5×10^6 cells/mL). The average percentages of cell growth at the i.p. and s.c. sites are summarized in Table 2. On the basis of these data, it was established that an acceptable starting density for Col2, Ishikawa, KB, KB-V1, Lu1, MCF-7, and SW626 cells with hollow fibers implanted in athymic mice was 10^6 cells/mL. For HUVEC and LNCaP cells, an acceptable starting density was 5×10^5 cells/mL, and for HL-60, Mel2, and P-388 cells, the value was 5×10^6 cells/mL (Table 2). In general, slow-growing cells must be seeded at a higher starting cell density so as to obtain reasonable optical density values,

Table 2. Growth Potential of Cells in the Hollow Fiber Model[a]

	cell density tested (cells/mL)						selected cell density[b]
	5×10^5		10^6		5×10^6		
cell line	i.p.	s.c.	i.p.	s.c.	i.p.	s.c.	i.p. & s.c.
HL-60	3466 ± 150	−583 ± 52	2131 ± 146	1244 ± 59	812 ± 3	570 ± 50	5×10^6
HUVEC	846 ± 81	1289 ± 140	1773 ± 150	976 ± 84	108 ± 10	96 ± 9	5×10^5
Ishikawa	2659 ± 151	1633 ± 140	660 ± 58	687 ± 85	637 ± 49	352 ± 33	10^6
KB	1380 ± 10	750 ± 43	637 ± 48	378 ± 45	110 ± 51	121 ± 37	10^6
KB-V1	923 ± 40	730 ± 65	619 ± 62	341 ± 5	141 ± 16	86 ± 9	10^6
LNCaP	1047 ± 90	1040 ± 77	1123 ± 99	262 ± 30	266 ± 14	99 ± 5	5×10^5
Lu1	2484 ± 101	247 ± 13	1944 ± 156	1291 ± 49	1900 ± 60	1260 ± 87	10^6
MCF-7	366 ± 39	447 ± 46	412 ± 26	324 ± 48	248 ± 25	252 ± 18	10^6
Mel2	6485 ± 122	3088 ± 101	1891 ± 124	2089 ± 98	883 ± 81	986 ± 65	5×10^6
P-388	9788 ± 141	6473 ± 653	4278 ± 112	3381 ± 104	339 ± 42	438 ± 41	5×10^6
SW626	1985 ± 60	629 ± 69	638 ± 64	441 ± 49	149 ± 59	141 ± 14	10^6

[a] The preliminary evaluation of each cell line consisted of preparing the hollow fiber samples at the indicated cell densities. Results are shown as the average percentage cell growth ± SE. Methodological details are described in the Experimental Section. [b] Cell density selected for hollow fiber studies.

whereas faster growing cells may plateau or decline if a higher starting cell density is adopted.

With KB and KB-V1 cells, at a density of 10^6 cells/mL, cells at the i.p. site increased 6−7-fold during the course of the experiment, and cells at the s.c. site increased 3−4-fold. This is preferable to a starting density of 5×10^6 cells/mL, since cell number did not significantly increase. With the lower starting cell density, large increases were observed at the i.p and s.c. sites, but this may lead to oversensitivity during drug treatment. For similar reasons, a starting cell density of 10^6 cells/mL was also selected for SW626 cells. With HL-60, Mel2, and P-388 cells, cell densities of 5×10^5 and 10^6 cells/mL were not selected, since 18−97-fold increases in growth were observed. With MCF-7 cells, based on the cell growth percentage, cell densities of 5×10^5 or 10^6 cells/mL were acceptable, but due to the slow-growing nature of the cells, 10^6 cells/mL was adopted to ensure reasonable optical density values. As for LNCaP cells, 5×10^6 or 10^6 cells/mL could not be used due to the low percentage of cell growth observed at the s.c. site. With Ishikawa cells, cell densities of 10^6 or 5×10^6 cells/mL were acceptable, and 10^6 cells/mL was selected. In the case of HUVEC cells, since a low percentage of cell growth was observed at 5×10^6 cells/mL and a high percentage of cell growth was observed at 10^6 cells/mL, a density of 5×10^5 cells/mL was adopted. For Lu1 cells, none of the cell densities were ideal, but 10^6 cells/mL was selected, as with 5×10^5 cells/mL, increases of only 2-fold were observed at the s.c. site, and with 5×10^6 cells/mL, the increases were similar to those observed at 10^6 cells/mL. With Col2 cells, 10^6 cells/ml was selected, as the i.p. and s.c. cells increased 8- and 5-fold, respectively, which is ideal.

Next, since many of the test materials to be evaluated are not readily soluble in aqueous solution, administration is problematic. A variety of methods have been investigated to modify the dissolution characteristics of poorly water-soluble drugs.[8] One versatile technique involves the formation of coprecipitates with pharmacologically inert, polymeric materials such as poly(vinylpyrrolidone) (PVP).[9,10] PVP coprecipitation has been used to enhance the dissolution characteristics of drugs such as hydrochlorothiazide,[11] reserpine,[12−14] allopurinol,[15] and digitoxin.[16] Thus, in our experimental design, as a standard practice, drugs were coprecipitated with PVP.

To assess the general performance of the hollow fiber model, some pilot studies were performed with paclitaxel (**1**), which has been previously studied in xenograft models.[17−20] When tested with mammalian cells in culture, this substance showed general toxicity, with the exception of KB-V1 cells, which were resistant (Table 1). With the in vivo hollow fiber model, as a positive control, paclitaxel was tested using KB, KB-V1 and SW626 cells, at doses of 4.39, 8.78, 17.6, and 35.1 μmol/kg (Table 3). With KB cells (Figure 2A), relative to the phosphate-buffered saline (PBS) control, paclitaxel treatment caused 70.2−85.3% growth inhibition at the i.p. compartment of mice, and no significant growth inhibition at the s.c. compartment. Using KB-V1 cells (Figure 2B), paclitaxel treatment groups showed no significant growth inhibition at the i.p. or the s.c. compartments. With SW626 cells (Figure 2C), paclitaxel caused 98.7−100% growth inhibition at the i.p. compartment, but no significant effect was noted at the s.c. compartment. No significant loss in body weight was observed with any of the treatment groups.

The i.p. responses in the hollow fiber model were consistent with in vitro studies, but the lack of response at the s.c. site was not consistent. Thus, employing similar experimental conditions, compound **1** was tested with an in vivo xenograft model (Figure 5A) in which KB cells were implanted. Overall, no significant difference was observed among the various treatment and control groups, which was consistent with the results observed at the s.c. compartment in the hollow fiber test. A statistical analysis of each treatment group is given in Table 4. In all cases, no differences were noted. Since KB cells were transplanted subcutaneously in the xenograft model, the therapeutic effects of compound **1** in this model appear to correlate with results obtained at the s.c. site in the hollow fiber model. It was not possible to increase the dose since treatment with 35.1 μmol/kg was lethal to half of the mice at day 34.

Dioscin (**2**) was isolated from *Cestrum pallidissimum* Backer (Solanaceae) as described in the Experimental Section. This compound has also been found in plants from the Agavaceae,[21] Arecaceae,[22] Dioscoreaceae,[23] Fabaceae,[24] Liliaceae,[25] Solanaceae,[26] Trilliaceae,[27] Zingiberaceae,[28] and Zygophyllaceae.[29] It has been reported that **2** functions as an immunomodulator[30] and an inhibitor of neoplasms,[31,32] xanthine oxidase,[33] cell proliferation,[30] and cyclic AMP phosphodiesterase.[34] It was also suggested that **2** showed antiviral,[35] antiflammatory,[35] antimutagenic,[36] antiyeast,[37] antifungal, and cytotoxic activities.[38] When tested against cultured cells (Table 1), **2** showed general cytotoxicity with Lu1, SW626, LNCaP, and Mel2 cells and moderate growth inhibitory activity with KB, MCF-7, and P-388 cells. In hollow fiber tests performed with **2**, doses of 57.5 and 144 μmol/kg were lethal to mice. Therefore, doses of 7.13, 14.3, and 28.5 μmol/kg were selected, and tests were performed

Table 3. Summary of Growth Inhibitory Effects of Compounds **1**−**6** in the Hollow Fiber Model[a]

compound	dose tested (μmol/kg)[b]	cell line evaluated	i.p. inhibition (%)	s.c. inhibition (%)	body weight change[e]
1	4.39, 8.78, 17.6, 35.1	KB	70.2−85.3[c]	19.5−27.0[d]	(+1.7)−(+1.1)
		KB-V1	0−3.5[d]	13.1−16.1[d]	
		SW626	98.7−100[c]	0−22.2[d]	
2	7.13, 14.3, 28.5	Lu1	24.6−95.5[c]	0−1.8[d]	(+0.80)−(−0.65)
		SW626	4.1−49.5[c]	8.7−26.6[d]	
		LNCaP	36.9−72.3[c]	35.9−44.7[c]	
		Mel2	2.3−25.9[c]	0[d]	
		KB	79−100[c]	3.6−47.7[d]	
		MCF-7	0−4.9[d]	7.3−9.9[d]	
		P-388	0−1.6[d]	0[d]	
3	72.6, 145.3, 290.7	Lu1	0−24.5[d]	0[d]	(+0.74)−(−0.85)
		SW626	0−91.7[c]	16.3−60.2[c]	
		LNCaP	50.6−57.6[c]	0[d]	
		Mel2	15.5−85.6[c]	11.1−60.3[c]	
		KB	21.6−100[c]	5.7−45.3[c]	
		MCF-7	34.4−88.7[c]	0−35.5[d]	
		P-388	0−40.4[c]	0[d]	
4	553.1	KB	0[d]	0[d]	+ 0.50
5	128, 256, 512	SW626	0−5.2[d]	10.3−26.2[d]	(+0.30)−(+0.45)
6	15.5, 30.9, 61.9, 124	Lu1	0[d]	0[d]	(+0.85)−(−0.40)

[a] Animals were treated with PBS (control) or the indicated doses of test substance, once daily by intraperitoneal injection from day 3−6 after implantation. On day 7, mice were sacrificed, and fibers were retrieved and analyzed as described in the Experimental Section. Results are shown as the average percentage cell growth relative to control. [b] Doses were selected as described in the text. [c] Statistical significance achieved with one or more dose levels. [d] Statistical significance not achieved with one or more dose levels. [e] Body weight reductions of ≤10% total body weight (i.e., reductions of less than ∼2 g) are deemed acceptable for this test.[5]

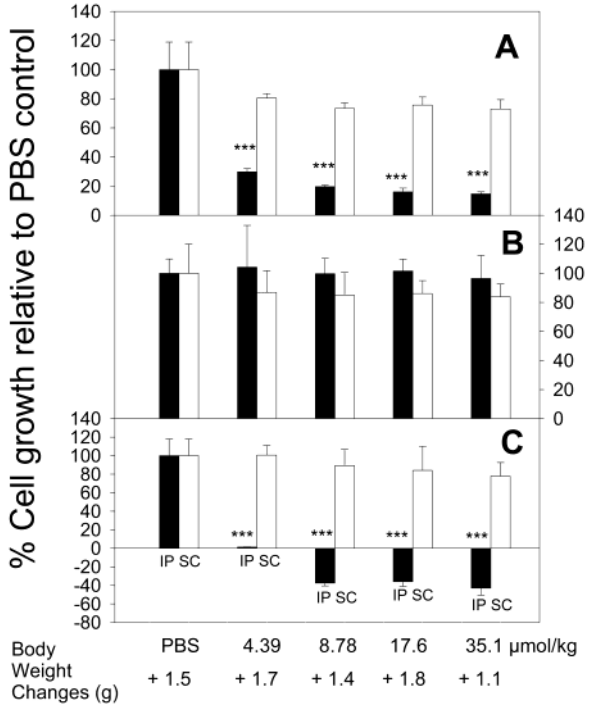

Figure 2. Effect of **1** on the growth of KB (panel A), KB-V1 (panel B), and SW626 (panel C) cells implanted at the i.p. (solid column) and the s.c. (open column) compartments of NCr *nu*/*nu* mice. The animals were treated with PBS (control) or the indicated doses of **1** once daily by intraperitoneal injection from day 3−6 after implantation. On day 7, mice were sacrificed, and fibers were retrieved and analyzed as described in the Experimental Section. Results are shown as the average percentage cell growth relative to control, ± SE (bars). Changes in mouse body weight at the end of the experiment are listed at the bottom of the figure. ***, treatment groups were significantly different from the control group (p <0.0001) using Student's t-test, with n = 6 for the control group and n = 3 for treatment groups.

with Lu1, SW626, LNCaP, Mel2, KB, MCF-7, and P-388 cells. Consistent with in vitro data (Table 1), **2** also showed significant growth inhibitory effects with Lu1, SW626, LNCaP, Mel2, and KB cells, but no significant growth inhibitory effects with MCF-7 and P-388 cells in the hollow fiber model (Figure 3, Table 3). With Lu1 cells (Figure 3A), **2** showed dose-dependent growth inhibitory effects (24.6−95.5%) at the i.p. compartment of mice. Relative to the PBS control, there were no significant growth inhibitory effects observed at the s.c. site. Similarly, with SW626 cells (Figure 3B), **2** demonstrated growth inhibitory effects (4.1−49.5%) at the i.p. compartment, but no significant inhibition was observed at the s.c. site. With LNCaP cells (Figure 3C), **2** showed dose-dependent growth inhibitory effects (36.9−72.3%) at the i.p. compartment of mice; at the s.c site, growth inhibition up to 44.7% was observed. With Mel2 cells (Figure 3D), at the i.p. site, growth inhibition was noted only at the highest concentration tested (25 μmol/kg), and no effect was observed at the s.c. site. With KB cells (Figure 3E), **2** showed dose-dependent growth inhibitory effects ranging from 79 to 100% at the i.p. site, but no significant inhibition was observed at the s.c. site. Compound **2** did not show significant growth inhibitory effects with MCF-7 (Figure 3F) or P-388 cells (Figure 3G). No significant loss in body weight was observed with any of the treatment groups.

Compound **2** was then evaluated with KB cells in a xenograft experiment (Figure 5B). In general, no significant difference was observed among the different treatment groups relative to the control group, which was consistent with the lack of response observed in the s.c. compartment with the hollow fiber test. The statistical analysis of each treatment group is given in Table 4.

13-Methoxy-15-oxozoapatlin (**3**) was isolated from *Parinari curatellifolia* Benth. (Chrysobalanaceae) through bioactivity-guided fractionation.[39] Compound **3** was found to demonstrate a broad spectrum of cytotoxic activity against a panel of cultured human cancer cell lines.[39] As shown in Table 1, **3** showed general toxicity against all of the tested cells. With the in vivo hollow fiber model, the effects of **3**, at doses of 72.6, 145.3, and 290.7 μmol/kg, were tested with Lu1, SW626, LNCaP, Mel2, KB, MCF-7, and P-388 cells. The upper limit of the dose was based on maximum solubility in vehicle. Consistent with in vitro data, when **3** was tested with cells in the hollow fiber model, it also demonstrated significant growth inhibitory effects with cells implanted at the i.p. compartment (except with Lu1 cells) (Figure 4, Table 3). In some cases, however, activity

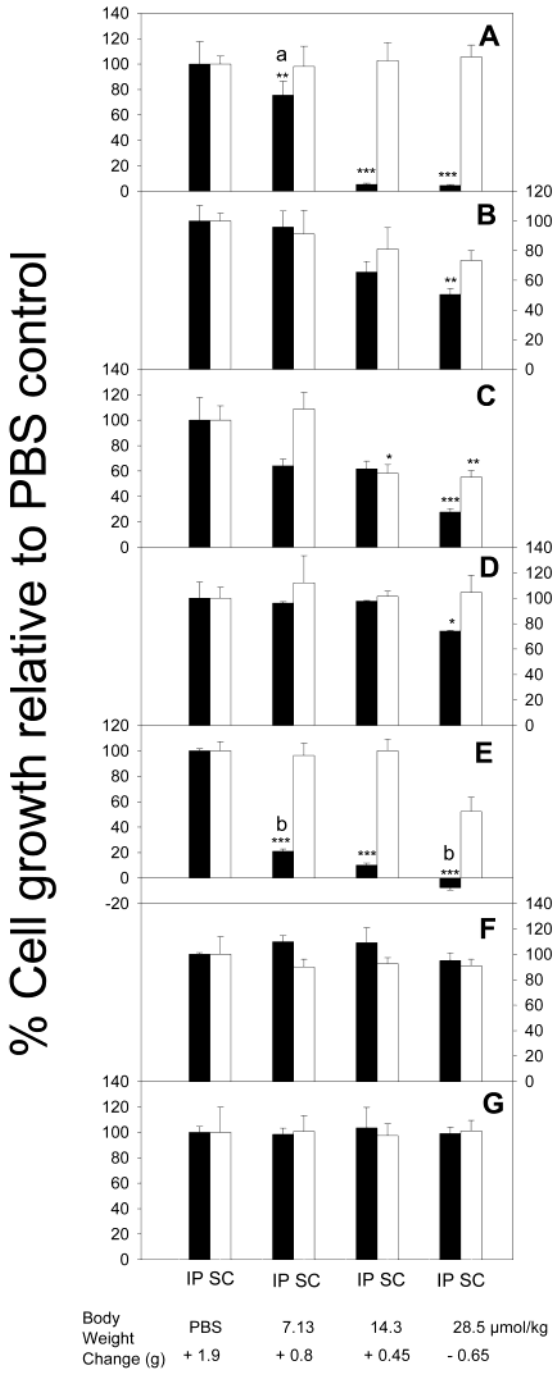

Figure 3. Effect of **2** on the growth of Lu1 (panel A), SW626 (panel B), LNCaP (panel C), Mel2 (panel D), KB (panel E), MCF-7 (panel F), and P-388 (panel G) cells implanted at the i.p. (solid column) and the s.c. (open column) compartments of NCr *nu*/*nu* mice. The animals were treated with PBS (control) or the indicated doses of **2** once daily by intraperitoneal injection from day 3−6 after implantation. On day 7, mice were sacrificed, and fibers were retrieved and analyzed as described in the Experimental Section. Results are shown as the average percentage of cell growth relative to control, ± SE (bars). Changes in mouse body weight at the end of the experiment are listed at the bottom of the figure. ***, **, and *, treatment groups were significantly different from the control group (p <0.0001, p <0.001, and p <0.01, respectively); a, there were significant differences with other drug treatment groups (p <0.001); b, there were significant differences between two drug treatment groups (p <0.001) using Student's t-test, with n = 6 for the control group and n = 3 for treatment groups.

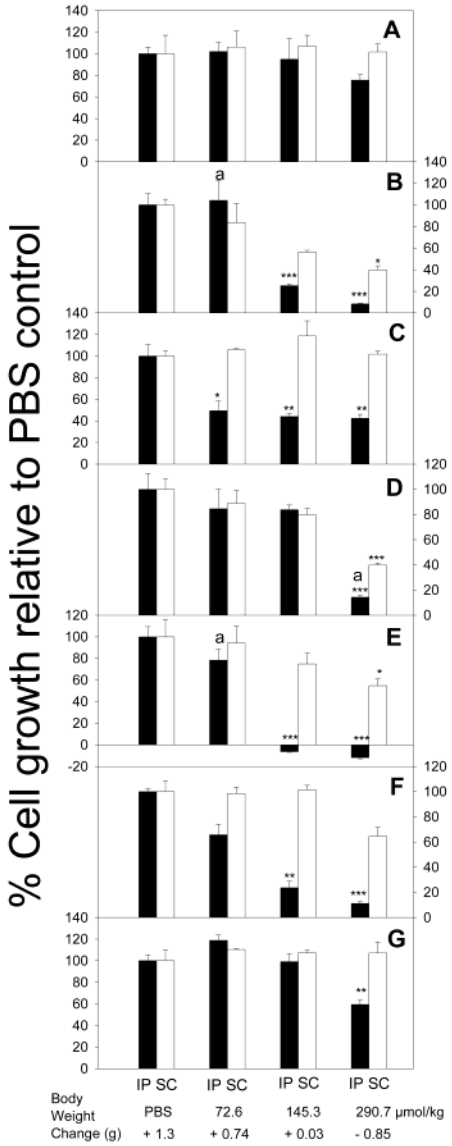

Figure 4. Effect of **3** on the growth of Lu1 (panel A), SW626 (panel B), LNCaP (panel C), Mel2 (panel D), KB (panel E), MCF-7 (panel F), and P-388 (panel G) cells implanted at the i.p. (solid column) and the s.c. (open column) compartments of NCr *nu*/*nu* mice. The animals were treated with PBS (control) or the indicated doses of **3** once daily by intraperitoneal injection from day 3−6 after implantation. On day 7, mice were sacrificed, and fibers were retrieved and analyzed as described in the Experimental Section. Results are shown as the average percentage cell growth relative to control, ± SE (bars). Changes in mouse body weight at the end of the experiment are listed at the bottom of the figure. ***, **, and *, treatment groups were significantly different from the control group (p <0.0001, p <0.001, and p <0.01, respectively); a, there were significant differences with other drug treatment groups (p <0.0001) using Student's t-test, with n = 6 for the control group and n = 3 for treatment groups.

was also observed with cells implanted at the s.c. compartment. For example, with SW626 cells (Figure 4B), **3** showed growth inhibitory effects at both the i.p. and the s.c. sites, with inhibitory percentages that ranged up to 91.7% at the i.p. site and 16.3−60.2% at the s.c. site. With LNCaP cells (Figure 4C), **3** demonstrated growth inhibitory effects (50.6−57.6%) at the i.p. site, but not at the s.c. site. For Mel2 cells (Figure 4D), **3** demonstrated dose-dependent growth inhibitory effects at both compartments, with inhibitory percentages that ranged from 15.5 to 85.6% at the i.p. site and 11.1−60.3% at the s.c. site. Similarly, **3** demonstrated dose-dependent growth inhibitory effects with KB cells (Figure 4E) at both sites, with inhibitory percentages that ranged from 21.6 to 100% at the i.p. site and 5.7 to 45.3% at the s.c. site. Growth inhibitory effects were also observed with MCF-7 cells (Figure 4F), ranging from 34.4 to 88.7% at the i.p. site, but no effect was observed at the s.c. site. Finally, **3** showed a 40.4% growth

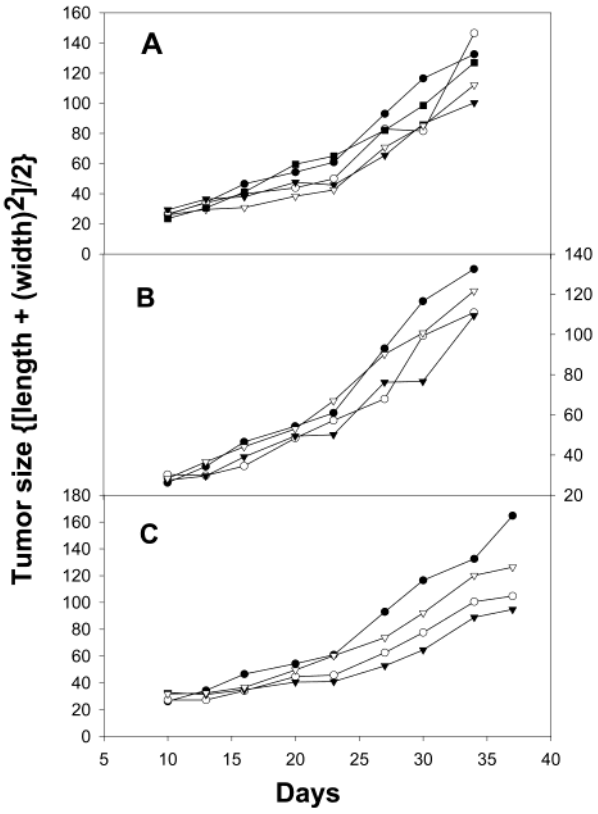

Figure 5. Effects of compounds **1** [panel A: PBS (●); 35.1 μmol/kg (○); 17.6 μmol/kg (▲); 8.78 μmol/kg (△); and 4.39 μmol/kg (■)], **2** [panel B: PBS (●); 28.5 μmol/kg (○); 14.3 μmol/kg (▲); and 7.13 μmol/kg (△)], and **3** [panel C: PBS (●); 290.7 μmol/kg (○); 145.3 μmol/kg (▲); and 72.6 μmol/kg (△)] on tumor growth. KB cells were transplanted subcutaneously (s.c.) unto the right flank region of female athymic NCr *nu*/*nu* mice on day zero. Mice were treated intraperitoneally with either control vehicle (PBS) or compounds (**1**−**3**) on days 10, 13, 16, and 20. Tumor size was measured twice weekly during the study. Individual tumor volumes (V) were calculated by the formula $V = [\text{length} + (\text{width})^2]/2$.

Table 4. Evaluation of Compounds **1**−**3** with Athymic Mice Innoculated with KB Cells[a]

compound	number of nude mice per group	dose (μmol/kg)	deaths	BWC (%)[b]	statistical significance[c]
PBS	6		0	+7.4	
1	6	4.39	0	+9.4	$p > 0.99$
	6	8.78	0	+6.7	$p = 0.85$
	6	17.6	0	+8.2	$p = 0.83$
	6	35.1	3	+7.8	$p = 0.85$
2	6	7.13	0	+7.1	$p = 0.96$
	6	14.3	0	+8.4	$p = 0.51$
	6	28.5	0	+8.6	$p = 0.64$
3	6	72.6	0	+7.9	$p = 0.51$
	6	145.3	0	+7.6	$p = 0.02$
	6	290.7	0	+6.6	$p = 0.06$

[a] Animals were treated with PBS (control) or the indicated doses of test substance, intraperitoneally, on days 10, 13, 16, and 20 after inoculation of KB cells. Tumor size was measured twice weekly. [b] The body weight was determined twice weekly, and changes were expressed as a percentage (body weight change, BWC). [c] Tumor sizes of treatment groups were compared with the control group, and statistical significance (p) was evaluated using Tukey's Studentized Range test.

inhibitory effect with P-388 cells at the i.p. site with a dose of 290.7 μmol/kg (Figure 4G), with no activity at the s.c. site. No significant loss in body weight (≥10%)[7] was observed with any of the treatment groups.

In sum, **3** mediated a general response with cultured cells and with various cell types (except Lu1 cells) implanted at the i.p. compartment in the hollow fiber model. At the s.c. site, unlike compound **2**, **3** showed significant growth inhibitory effects with SW626, Mel2, and KB cells. Compound **3** was further studied in the xenograft model with KB cells (Figure 5C). Generally, treatment groups were significantly different from the control group. As indicated in Table 4, doses of 145.3 and 290.7 μmol/kg achieved statistical significance ($p = 0.02$, 0.06, respectively), whereas in the hollow fiber test, only the dose of 290.7 μmol/kg achieved statistical significance ($p < 0.01$) at the s.c. site. In previous studies, compound **3** did not demonstrate in vivo antitumor activity with KB cells transplanted in athymic mice, when tested at a maximum tolerated dose of 261.6 μmol/kg, administered i.p. on days 1, 5, 9, with 10% DMSO (dimethyl sulfoxide), and with 1% Tween as vehicle.[39] These differences may be due to variations in the dose regimens and formulations.

Ochraceolide A (**4**) came to our attention because it was active as a farnesyl protein transferase inhibitor (IC_{50} = 2.2 μM).[40] Earlier, compound **4** was isolated in our laboratories as a cytotoxic agent against the P-388 cell line from a nonpolar extract derived from *Kokoona ochracea* (Elm.) Merrill stem bark,[41] and this sample was used in the present investigation. The compound exhibited significant cytotoxic activity with cultured P-388 cells ($IC_{50} = 0.58$ μM), but was at least 10-fold less active with various human tumor cell lines.[41] As currently demonstrated, it was modestly cytotoxic against various cell lines (Table 1). In the hollow fiber model at a dose of 553.1 μmol/kg, the moderate cytotoxic effect of **4** observed with cultured KB cells (IC_{50} = 20.3 μM) did not translate to a positive response at either the i.p. or s.c. sites (Table 3). This might be due to differences between in vitro and in vivo systems, including drug metabolism, or simply reflect the weak activity mediated by the compound.

Compound **5** (α-lapachone) was isolated as a cytotoxic agent by activity-guided fractionation using the BC1 human breast and Lu1 human lung cancer cell lines from the roots of *Ekmanianthe longiflora* (Griseb.) Urb.[42] It was reported recently that **5** inhibits initial noncovalent binding of topoisomerase II to DNA and induces religation of DNA breaks.[43] When evaluated with cultured cells, **5** exhibited a broad cytotoxic response with the panel (Table 1), but the response was not generally strong. For the hollow fiber model, SW626 cells (in vitro $IC_{50} = 12.4$ μM) were selected, and **5** was tested at doses of 128, 256, and 512 μmol/kg. There was no significant loss of body weight (Table 3), and no significant effect on cell growth.

The furanoquinone **6** was isolated from the roots of *Ekmanianthe longiflora* in the same investigation as **5**.[42] It was found by others to possess pronounced activity against the parasites *Trypanosoma brucei brucei* and *Trypanosoma brucei rhodesiense*.[44] When tested with cultured cells, **6** showed broad cytotoxic activity (Table 1). In the hollow fiber model, compound **6** was tested with Lu1 cells ($IC_{50} = 0.826$ μM). A dose of 516 μmol/kg was lethal to mice, and a dose of 252 μmol/kg caused significant body weight loss. At doses of 15.5, 30.9, 61.9, and 124 μmol/kg, no significant body weight loss was observed, but the compound did not mediate growth inhibitory effects with Lu1 cells (Table 3), which is consistent with the results of Peraza-Sánchez et al., where the compound was found to be inactive when tested at 297 μmol/kg/injection, administered intraperitoneally in an in vivo mouse P-388 leukemia system.[42]

As summarized above, using the hollow fiber model, relatively poor responses were observed at the s.c. site with

several compounds that were active in cell culture and/or the i.p. site. In this regard, Phillips et al.[45] have raised concerns about the efficiency of drug delivery and its subsequent effects on chemosensitivity. They found that responses at the i.p. site might be expected to reflect in vitro chemosensitivity, as it is unlikely that drug delivery problems would occur after an i.p. drug administration. Responses at the s.c. site, however, after i.p. drug administration, may be absent since the present hollow fiber assay protocol would not enable angiogenesis. The current work does not support these generalizations. Compounds **2**−**6** were all active in cell culture, but only compounds **2** and **3** were active in the hollow fiber model at the i.p. site. Moreover, in addition to mediating a response at the i.p. site, with at least one cell line, both of these compounds mediated responses at the s.c. site. These results indicate that angiogenesis is not a prerequisite for activity, and these agents are capable of mediating responses at distal sites.

Nonetheless, as suggested by Casciari et al.,[6] there are two disadvantages of using the hollow-fiber model in drug-screening studies. First, tumor growth is limited by the geometric constraint of the fiber wall. Second, the fiber wall constitutes an artificial barrier that separates the tumor cells from their surroundings. During the course of the present studies, the cytotoxic activities (IC_{50}) of compound **1**−**3** were found to be 0.02, 20.7, and 6.4 μM, respectively, with KB cells (Table 1). With the hollow fiber model, the growth inhibitory effects of compounds **1**−**3** were 85.3, 100, and 100%, respectively, at the i.p. site, and 27.0, 47.7, and 45.3%, respectively, at the s.c. site. Therefore, we further evaluated compounds **1**−**3** in the standard xenograft model with KB cells. In these tests, compound **3** achieved statistical significance, whereas compounds **1** and **2** were not active. Thus, the value of utilizing the hollow fiber model as a secondary discriminator is becoming apparent. Compounds that are not active in the hollow fiber model, even though they are active in cell culture, are assigned a low priority in terms of development. This pattern was observed with compounds **4**−**6** and illustrates the manner by which the hollow fiber model can help focus experimental efforts on agents with a greater likelihood of demonstrating clinical utility. On the other hand, compound **3** showed significant s.c. activity in the hollow fiber model, and subsequently was found active in the xenograft model. This could not have been predicted on the basis of in vitro data alone, nor on the basis of a hollow fiber response at the i.p. site. Fundamentally, it appears that positive responses in the s.c. site of the hollow fiber model should be given greatest attention.

Experimental Section

Chemicals and Test Compounds. All general chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. Polyvinylidene fluoride (PVDF) hollow fibers (500 000 Da molecular mass cutoff, 1.0 mm i.d.) were purchased from Spectrum Medical Industries (Luguan Hills, CA). Cell culture media and supplements were obtained from Life Technologies, Inc. (Grand Island, NY). MATRIGEL was purchased from Becton Dickinson Labware (Belford, MA). Dioscin (**2**) was isolated from *Cestrum pallidissimum* Backer (Solanaceae) as described below, 13-methoxy-15-oxozoapatlin (**3**) was isolated from *Parinari curatellifolia* Benth. (Chrysobalanaceae),[39] ochraceolide A (**4**) was isolated from nonpolar extracts derived from *Kokoona ochracea* (Elm.) Merrill (Celastraceae) stem bark,[40] and naphthoquinones **5** and **6** were isolated from the roots of *Ekmanianthe longiflora* (Griseb.) Urb (Bignoniaceae).[42] For hollow fiber studies, compounds and extracts were coprecipitated with PVP to increase solubility and then suspended in PBS.[46]

Plant Material. The leaves of *Cestrum pallidissimum* Backer were collected at Desa Batulawang, Kecamatan Pacet, Kabupaten Cienjur, West Java, Indonesia, in June 1996 (*S. Riswan & J. J. Afriastini J-026*). A voucher specimen representing this collection (A3876) is deposited at the Field Museum of Natural History (accession number 2192971).

Extraction and Isolation of Dioscin (2). The dried, powdered leaves (770 g) of *Cestrum pallidisimum* were extracted two times overnight with MeOH. After filtration, the resultant extracts were combined, concentrated, and then diluted with H_2O to afford an aqueous MeOH (1:9 v/v, 1 L) solution, which was defatted with petroleum ether (5 × 700 mL). The methanolic layer was evaporated and the residue partitioned between $CHCl_3$ and MeOH (1:1). The combined $CHCl_3$ layers were washed with 1% NaCl and concentrated to give an extract (10.2 g).

A portion (10.0 g) of the $CHCl_3$ extract was subjected to column chromatography over Si gel, using Lu1 cells to monitor the fractionation. Elution with mixtures of $CHCl_3$ and MeOH of increasing polarity afforded 10 major fractions. Dioscin (**2**) (200 mg) was isolated from fraction 7 by elution with the mixtures of $CHCl_3$ and MeOH (3:1) and was purified by further Si gel column chromatography by elution with the mixtures of $CHCl_3$, MeOH, and H_2O (8:2:0.2). The structure of dioscin (**2**) was determined by data comparison (mp, $[\alpha]_D$, UV, IR, ^{1}H NMR, ^{13}C NMR, FABMS) with literature values.[47]

Cell Cultures. Human promyelocytic leukemia HL-60 cells, human oral epidermoid carcinoma KB cells, human prostate carcinoma LNCaP cells, human breast carcinoma MCF-7, mouse lymphoid neoplasm P-388 cells, and human ovarian adenocarcinoma SW626 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD). KB-V1 cells were supplied by Dr. I. B. Roninson (Department of Molecular Genetics, University of Illinois at Chicago, Chicago, IL). The BC1 human breast cancer cell line, the Col2 human colon carcinoma cell line, the Lu1 human lung carcinoma cell line, and the Mel2 human melanoma cell line were obtained from the Department of Surgical Oncology, University of Illinois at Chicago, Chicago, IL. Human umbilical vein endothelial HUVEC cells were purchased from CLONTECH (CLONTECH, Palo Alto, CA), and Ishikawa human endometrial carcinoma cells were supplied by Dr. Richard Hochberg of the Department of Obstetrics and Gynecology at Yale University, New Haven, CT. BC1 and Col2 cells were maintained in MEME medium. HL-60 cells were maintained in RPMI medium. HUVEC cells were maintained in EGM-2 bulletkit medium. Ishikawa cells were maintained in DMEM/F-12 medium. KB cells were maintained in DMEM medium. KB-V1 cells were grown in the same medium as KB cells, which was further supplemented with vinblastine (1 μg/mL). LNCaP cells were maintained in RPMI1640 medium with hormone-free 10% heat-activated FBS (fetal bovine serum) supplemented with 0.1 nM testosterone. Lu1 cells were cultured in MEME medium. MCF-7 cells were maintained in MEME medium containing 10 mg/L of insulin. Mel2 cells were maintained in MEMH medium. P-388 cells were maintained in Fisher's medium. SW626 cells were maintained in RPMI medium. In each case, PSF (100 units/mL penicillin G, 100 μg/mL streptomycin sulfate, 250 ng/mL amphotericin B) was added. All media were supplemented with 10% heat-inactivated FBS.

Cytotoxic Potential. The cytotoxic potential of the test substances with cultured cells was determined as described previously.[42] Briefly, various concentrations of test compounds (dissolved in 10 μL of 10% DMSO) were transferred to 96-well plates, and 190 μL of the cell suspensions was added to each well. The plates were then incubated for 72 h at 37 °C (100% humidity with a 5% CO_2 atmosphere in air), and 50−100 μL of cold 10−50% aqueous trichloroacetic acid was added to the growth medium in each well to fix the cells. The cultures were incubated at 4 °C for 30 min, washed, air-dried, stained with sulforhodamine B solution, and washed with 1% acetic acid.

Finally, 200 μL of 10 mM Tris base was added to each well, and the optical densities were determined at 515 nm utilizing an ELISA plate reader. In each case, a zero-day control was performed by adding an equivalent number of cells to several wells and incubating at 37 °C for 30 min, and processing as described above. Optical density values obtained with the zero-day control were subtracted, and cell survival, relative to control (solvent-treated) cultures, was calculated.

In Vivo Hollow Fiber Test. The in vivo hollow fiber test was performed using a literature procedure with some modification.[5] Confluent monolayers of the cells were harvested, collected by centrifugation, and resuspended in conditioned medium at a concentration of 10^6 or 5×10^5 cells/mL. Fibers filled with cells were incubated in six-well plates overnight at 37 °C in a 5% CO_2 atmosphere. Female athymic NCr *nu*/*nu* mice (5−6-weeks-old) were obtained from the National Cancer Institute−Frederick Cancer Research Facility (Frederick, MD). Each mouse hosted up to six fibers, which were cultured in two physiologic compartments. For intraperitoneal implants, a small incision was made through the skin and musculature of the dorsal abdominal wall of the mouse, the fiber samples were inserted into the peritoneal cavity in a craniocaudal direction, and the incision was closed with skin staples. For subcutaneous implants, a small skin incision was made at the nape of the neck to allow insertion of an 11-gauge tumor implant trocar. The trocar, containing the hollow fiber samples, was inserted caudally through the subcutaneous tissues and fibers were deposited during withdrawal of the trocar. The incision was closed with skin staples.

In preliminary studies, cell growth was assessed with fibers containing 5×10^5, 10^6, or 5×10^6 cells/mL. For treatment protocols, drugs were coprecipitated with PVP (MW 360,000, Sigma)[46] to increase solubility and then dissolved in PBS. Mice were randomized into PBS vehicle control groups (six mice per group) and drug treatment groups (three mice per group for each dose tested). Test compounds were administrated once daily by intraperitoneal injection from day 3−6 after implantation. Body weights were determined daily.

On day 7, mice were anesthetized with an i.p. injection of a mixture of ketamine (13.3 mg/100 g body weight) and xylazine (1.3 mg/kg body weight) to retrieve the fibers. The fibers were placed into six-well plates, each well containing 2 mL of fresh, prewarmed culture medium (with 20% calf serum or FBS), and allowed to equilibrate for 30 min at 37 °C. To define the viable cell mass contained within the intact hollow fibers, a MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye conversion assay was used. Briefly, 1 mL of prewarmed culture medium (with 20% calf serum or FBS) containing 1 mg MTT/mL was added to each dish. After incubating at 37 °C for 4 h, the culture medium was removed by aspiration, 2 mL of normal saline containing 2.5% protamine sulfate solution was added to each well, and the plates were stored at 4 °C for 24 h. Protamine sulfate solution was then removed by aspiration, 2 mL of fresh protamine sulfate solution was added for a second wash, and the plates were stored at 4 °C for at least 2−4 h. To assess the optical density of the samples, the fibers were transferred to 24-well plates, cut in half, and allowed to dry overnight. The formazan was extracted from each sample with DMSO (250 μL/well) for 4 h at room temperature on a rotation platform. Aliquots (150 μL) of extracted MTT formazan were transferred to individual wells of 96-well plates and assessed for optical density at a wavelength of 540 nm. The effect of the treatment regimen was determined by the net growth percentage of the cells relative to changes in body weight.

In Vivo Xenograft Test. The in vivo xenograft test was performed using a literature procedure with some modification.[48] Confluent monolayers of the KB cells were harvested, collected by centrifugation, and suspended in MATRIGEL (5×10^5 cells/0.19 mL MATRIGEL, using a precooled pipet, syringe, and needle as required). Cell suspensions (0.19 mL) were transplanted subcutaneously (s.c.) into the right flank region of female athymic NCr *nu*/*nu* mice (the same characteristics as those used for the in vivo hollow fiber tests) on day zero. Mice were randomly distributed to the experimental groups. When the tumors reached a palpable mass at day 10, treatment was initiated. Drug preparation was the same as described for the in vivo hollow fiber test. Mice were treated intraperitoneally with either PBS (control) or test compounds on days 10, 13, 16, and 20. Tumor size was measured twice weekly with a digital caliper in two dimensions. Individual tumor volumes (V) were calculated by the formula $V = [\text{length} + (\text{width})^2]/2$ and used for evaluating the efficacy of the compounds. Body weights were determined twice weekly and expressed as percent change after initiation of treatment.

Acknowledgment. Support of this work was provided by Grant U19 CA52956, funded by the National Cancer Institute. The authors are grateful to I. B. Roninson (Department of Molecular Genetics, University of Illinois at Chicago, Chicago, IL) for supplying KB-V1 cells, Jacinto B. Regalado (Field Museum of Natural History, Chicago, IL), for taxonomic assistance, and Richard B. Dvorak (Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago, Chicago, IL) for the FABMS of **2**. We thank the Nuclear Magnetic Resonance Laboratory of the Research Resources Center, University of Illinois at Chicago, for the provision of certain NMR spectral facilities used in this investigation.

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NP010322W