**In Vitro and In Vivo Anticancer Activities of Synthetic Macrocylic Ketone Analogues of Halichondrin B**


**INTRODUCTION**

Halichondrin B is a highly potent anticancer agent originally found in marine sponges, including *Halichondria okadai* (1, 2), *Astinella sp.* (3), *Phakellia carteri* (4), and *Lissondendryx sp.* (5). In 1986, halichondrin B was shown to have remarkable in vitro and in vivo anticancer activities against murine melanomas and leukemias (2). Subsequent studies by several groups (6–9) confirmed the sub-nm in vitro potency of halichondrin B and identified a tubulin-based antimitotic mechanism. Although halichondrin B is classified as a tubulin depolymerizer (similar to *Vinca* alkaloids, dolastatins, cryptophycin, and so on), the patterns of its interaction with tubulin are highly specific and place it in its own unique class within this group (6–9). Although speculative, unique interactions with tubulin may help explain the marked in vivo anticancer potency of halichondrin B, now well documented in several human tumor models including melanoma, osteogenic sarcoma, lung cancers, and colon cancers (10). More importantly, unique tubulin interactions relative to existing tubulin-based drugs suggest the possibility of unique tumor specificities or other desirable clinical characteristics.

Although the extraordinary potency of halichondrin B generated considerable interest for development, limited availability of the natural product severely restricted such efforts. However, the existence of a synthetic route for halichondrin B (11) and knowledge that its activity resides in the macrocyclic lactone C1-C38 moiety (3) (see also Refs. 12–14) have permitted development of structurally simplified synthetic analogues that retain the exceptional activity of the parent. In this study, we describe ER-076349 and ER-086526, two macrocyclic ketone analogues of halichondrin B with highly potent activities in vitro and in vivo. We show that these agents exert their effects via a tubulin depolymerizing antimitotic mechanism similar or identical to that reported for parental halichondrin B.

**MATERIALS AND METHODS**

**Chemical Synthesis.** Halichondrin B macrocyclic ketone analogues ER-076349 (NSC 707390) and ER-086526 (NSC 707389) were prepared as described previously (15). The biotinylated analogue ER-040798 and the biotinylated negative control ER-040792 were synthesized based on appropriate modifications to the published synthetic route for halichondrin B (11). The structures of halichondrin B and all of the synthetic analogues are presented in Fig. 1.

**Cells and Cell Culture.** The following human tumor cell lines were obtained from the American Type Culture Collection (Rockville, Maryland) and grown under American Type Culture Collection-recommended conditions: COLO 205 and DLD-1 colon cancer, HL-60 promyelocytic leukemia, U937 histiocytic lymphoma, and LNCaP and DU 145 prostate cancer. LOX human melanoma cells were obtained from the Division of Cancer Treatment-NCI Tumor Repository (Frederick, Maryland) and were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine. MDA-MB-435 human breast cancer cells were generously provided by Dr. Mary J. C. Hendrix (University of Iowa College of Medicine, Iowa City, Iowa) and were grown in DMEM (high glucose) supplemented with 10% heat-inactivated fetal bovine serum, 20 mM N-2-hydroxyethyl-piperazine-N’-2-ethanesulfonic acid and 1 mM sodium pyruvate.

All of the cell lines were grown at 37°C in a humidified atmosphere containing 5% CO2. Seeding densities for each cell line were empirically optimized for passaging twice weekly. Quantification of cell number was by hemocytometer counting, and viability determinations were made by standard trypan blue exclusion techniques. Routine harvesting of monolayer cultures was by standard trypsinization procedures.

**Cell Growth Inhibition Assays.** Cells were plated in 96-well plates at 7,500 cells/well (except LNCaP cells, which were at 10,000 cells/well) and grown in the continuous presence of test compounds for 4 days. For monolayer cultures (DU 145, LNCaP, LOX, and MDA-MB-435), growth was assessed using modifications (16) of a methylene blue-based microculture assay (17). For suspension cultures (HL-60, U937) or loosely adherent monolayers (COLO 205), growth was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide-based assay (18) modified as follows. After 4 days of incubation with test compounds, sterile-filtered 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma Chemical Co., St. Louis, MO) was added to each well (final concentration, 0.5 mg/ml), and plates were incubated at 37°C for 4 h. Acid-isopropanol (0.1 N HCl in isopropanol, 150 μl)
Fixed cells were then incubated with 1:50 diluted (v/v) in PBS anti-β-tubulin TUB 2.1 monoclonal antibody (Sigma) for 1 h at room temperature with rocking, followed by three 5-min washes with PBS and incubation with 1:50 diluted (v/v) in PBS FITC-conjugated goat F(ab')₂ antimum immunoglobulin (BioSource International, Camarillo, CA) for 1 h at room temperature with rocking. Cells were washed 3× with PBS, incubated with 2 μg/ml DAPI in PBS for 10 min at room temperature, followed by one 5-min wash with PBS and two 5-min washes with 2 ml Tris, 0.1 ml EDTA (pH 7.5). Chamber walls and gaskets were then removed, and the slides were air-dried. Coverslips were mounted with GelMount (Biomedia, Foster City, CA), and fluorescence microscopy was performed using a Nikon Labophot-2 microscope equipped with 100 × oil immersion objective and B-2A and UV-2A filter cubes for FITC and DAPI staining, respectively. Images were acquired with a Photometrics NU 2000 CCD camera and digitized using IPLab Spectrum software (Scanninglytics, Fairfax, VA).

Affinity Binding of Tubulin to Biotinylated Macrocyclic Analogue

ER-040798, UltraLink™ Immobilized NeutrAvidin Plus (Pierce Chemical Co., Rockford, IL) was charged with ER-040792, ER-040798 (135 μM final concentrations), or buffer alone at room temperature for 60 min in 20 mM Tris, 0.15 mM NaCl, 1 mM Na₂EDTA (pH 7.5; wash-elution buffer). After five washes in wash-eluted buffer, matrices were incubated in the same buffer with 375 μg/ml bovine brain tubulin (Molecular Probes, Eugene, OR) at room temperature for 30 min, followed by three washes in wash-elution buffer and elution with 20% acetonitrile (v/v in water). Eluted proteins were dried in a SpeedVac, resuspended in SDS sample buffer, and subjected to modified discontinuous SDS PAGE on 8% gels as described by Mathews (19) for separation of α- and β-tubulin subunits. Gels were silver-stained (20) or transferred to polyvinylidene difluoride membranes (Sigma) for Western immunoblotting (21) with subtype-specific anti-α- or anti-β-tubulin monoclonal antibodies (clones B-5-1-2 and TUB 2.1, respectively; Sigma) and horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin secondary antibodies (Amersham Corp., Arlington Heights, IL) using a chemiluminescence protocol (ECL Western Blotting Detection System; Amersham).

In Vitro Tubulin Polymerization Studies.

ER-076349, ER-086526, and vinblastine in 10 mM anhydrous DMSO stocks were diluted to 1% DMSO, 90% PEM. Substocks were made to final concentrations of 1, 10, 100, and 1000 μM; these were diluted into 100-μl volumes of 3.0 mg/ml bovine brain tubulin (Cytoskeleton, Inc., Denver, CO) in PEM buffer plus 1 mM ATP, 3% (v/v) glycerol to achieve desired test compound concentrations. The amount of 10% DMSO, 90% PEM was made up to 11 μl in all of the samples to achieve 1% final DMSO concentrations. Microtubule polymerization was initiated by raising the temperature from 4°C to 37°C over a 3-min period. Absorbance (A₃₄₀nm) was measured once/min for 60 min.

In Vivo Xenograft Studies.

Anticancer effects of ER-076349 and ER-086526 were evaluated in the MDA-MB-435 human breast cancer, COLO 205 human colon cancer, and LOX human melanoma xenograft models using 5–6-week-old female Swiss nude mice and in the NIH:OVCAR-3 human ovarian cancer model using 7-week-old female BALB/c nude mice. All of the studies using laboratory animals were approved either by the Eisai Research Institute (Andover, MA) or Tsukuba Research Laboratories (Tsukuba, Japan) Institutional Animal Care and Use Committees and adhered to all of the applicable institutional and governmental guidelines for the humane care and use of laboratory animals.

On day 0 of each experiment, mice received injections s.c. with 1 × 10⁶ cells (MDA-MB-435, COLO 205, and LOX) or 6 × 10⁵ cells (NIH:OVCAR-3; previously adapted for enhanced penetration via tumor passage in vivo). For COLO 205 and MDA-MB-435 models, mice received injections with 200 μl of test compound in saline on Monday/Wednesday/Friday i.p. (COLO 205) or i.v. (MDA-MB-435) schedules, respectively, beginning on day 13 for four weekly cycles. Treatment of mice bearing LOX tumors was by daily i.p. injection (200 μl) in saline on days 3–7 and 10–14 inclusive. In the NIH:OVCAR-3 model, test compounds were injected i.v. (200μg/20g body weight) in 4% DMSO/saline beginning on day 40 and continuing Monday/ Wednesday/Friday for three weekly cycles. Control groups in all of the studies received appropriate vehicle injections. Paclitaxel at its empirically determined MTD for each treatment regimen was included in all of the experiments and received appropriate vehicle injections. Paclitaxel at its empirically determined MTD for each treatment regimen was included in all of the experiments and received appropriate vehicle injections.
ER-076349 and ER-086526, respectively. C35, with hydroxyl and primary amine substituents for ER-076349 membered ring (Fig. 1). The two analogues themselves differ only at chondrin B is replaced by ketone, C31 methyl is replaced by methoxy, the tricyclic C29-C38 system is replaced by a single five-

ANTICANCER ACTIVITIES OF SYNTHETIC HALICHONDRIAN B ANALOGUES

In Vitro Inhibition of Human Tumor Cell Growth

Inhibition of Human Tumor Cell Growth in Vitro. More than 180 analogues of the macrocyclic C1-C38 moiety of halichondrin B were synthesized and evaluated in search of suitably active and chemically stable drug development candidates. This culminated in the identification of two simplified macrocyclic analogues, which time almost all of the living cells were blocked in G 2 -M. For 8 h, indicating apoptosis of cells after prolonged G 2-M blockage. The fact that both G1 and S phases became progressively depleted under continuous ER-076349 or ER-086526 exposure confirms normal movement of cells through these phases even in the presence of a drug. Thus, ER-076349 and ER-086526 are rapid and effective G2-M phase blockers that do not affect cell cycle progression through the G1 or S phases or the G1-S transition point. Similar G2-M

Table 1. Inhibition of human cancer cell growth by ER-076349, ER-086526, vinblastine, and paclitaxel

<table>
<thead>
<tr>
<th>Cell lineb</th>
<th>n</th>
<th>ER-076349</th>
<th>ER-086526</th>
<th>Vinblastine</th>
<th>Paclitaxel</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-435</td>
<td>4</td>
<td>0.14 ± 0.1</td>
<td>0.09 ± 0.01</td>
<td>0.59 ± 0.02</td>
<td>2.5 ± 0.34</td>
</tr>
<tr>
<td>COLO 205</td>
<td>2</td>
<td>0.41 ± 0.07</td>
<td>0.71 ± 0.05</td>
<td>2.4 ± 0.20</td>
<td>7.8 ± 1.5</td>
</tr>
<tr>
<td>DLD-1</td>
<td>3</td>
<td>0.75 ± 0.08</td>
<td>9.5 ± 1.0</td>
<td>7.3 ± 0.74</td>
<td>19 ± 2.3</td>
</tr>
<tr>
<td>DU 145</td>
<td>3</td>
<td>0.70 ± 0.06</td>
<td>0.91 ± 0.06</td>
<td>3.6 ± 0.92</td>
<td>9.4 ± 3.0</td>
</tr>
<tr>
<td>LNCaP</td>
<td>3</td>
<td>0.25 ± 0.02</td>
<td>0.50 ± 0.03</td>
<td>1.8 ± 0.15</td>
<td>3.8 ± 0.58</td>
</tr>
<tr>
<td>LOX</td>
<td>3</td>
<td>0.76 ± 0.11</td>
<td>1.4 ± 0.31</td>
<td>3.2 ± 0.39</td>
<td>7.3 ± 0.43</td>
</tr>
<tr>
<td>HL-60</td>
<td>2</td>
<td>0.41 ± 0.01</td>
<td>0.90 ± 0.24</td>
<td>2.6 ± 0.64</td>
<td>4.3 ± 1.3</td>
</tr>
<tr>
<td>U937</td>
<td>2</td>
<td>0.22 ± 0.04</td>
<td>0.43 ± 0.02</td>
<td>4.0 ± 2.0</td>
<td>3.9 ± 2.0</td>
</tr>
<tr>
<td>Average of eight cell linesc</td>
<td>8</td>
<td>0.45 ± 0.09</td>
<td>1.8 ± 1.1d</td>
<td>3.2 ± 0.70</td>
<td>7.3 ± 1.9</td>
</tr>
</tbody>
</table>

Footnotes:

a Growth inhibition determined under 4-day continuous exposure conditions as described in “Material and Methods.” Data shown are mean IC 50 values in nM from n separate experiments, each done with internal duplicates. Errors represent SE for n ≥ 3 or range for n = 2.

b Human cancer cell lines used: MDA-MB-435 breast cancer, COLO 205 and DLD-1 colon cancer, DU 145 and LNCaP prostate cancer, LOX melanoma, HL-60 promyelocytic leukemia, and U937 histiocytic lymphoma.

c Overall mean ± SE of the eight individual means from each cell line.

d ER-086526 mean excluding DLD-1 value (n = 7): 0.70 ± 0.16 nM.
blockage of U937 cells was observed previously using synthetic halichondrin B (24).

Disruption of Mitotic Spindles. The known anti-tubulin effects of halichondrin B (6–9) together with G2-M blockage by ER-076349 and ER-086526 led us to assess effects on mitotic spindles in DU 145 human prostate cancer cells (Fig. 4). In this study, cells in log phase growth were treated for 20 h with vehicle, 2.1 nM ER-076349, 2.7 nM ER-086526, 11 nM vinblastine, or 28 nM paclitaxel, representing IC50 levels on April 14, 2017. © 2001 American Association for Cancer Research.

To determine whether the tubulin-depolymerizing mechanism of halichondrin B (6) had been conserved during structural simplification to macrocyclic halichondrin analogues, effects of ER-076349 and ER-086526 on polymerization of bovine brain tubulin in vitro were assessed and compared with the known tubulin depolymerizer vinblastine. As shown in Fig. 6, ER-076349, ER-086526, and vinblastine inhibited both rates and extents of tubulin polymerization in dose-dependent manners. The inhibition of polymerization rates and extents was similar for each drug, with calculated IC50s for ER-076349, ER-086526, and vinblastine of 5.7 μM, 6.9 μM, and 1.8 μM for rate inhibition, and 5.5 μM, 6.0 μM, and 2.1 μM for extent inhibition, respectively. By either criterion, ER-076349 and ER-086526 were about 3–4-fold less effective as inhibitors of in vitro tubulin polymerization compared with vinblastine.

Although concentrations of all of the three drugs required to inhibit tubulin polymerization in vitro are several log orders higher than those that inhibit cell growth, such large discrepancies with tubulin-active agents may result from inhibited microtubule dynamics at low drug concentrations rather than net changes in cellular microtubule content which occur only at higher drug levels (25, 26). In addition, Vinca
alkaloids and paclitaxel are known to accumulate inside cells to high levels (25, 26); this may also occur with ER-076349 and ER-086526.

Inhibition of Human Tumor Xenograft Growth in Vivo. The abilities of ER-076349 and ER-086526 to inhibit tumor growth in vivo were tested in several human tumor xenograft models, including MDA-MB-435 breast cancer, COLO 205 colon cancer, LOX melanoma, and NIH:OVCAR-3 ovarian cancer (Fig. 7). In all of the cases, treatment with ER-076349 and ER-086526 in the 0.05–1 mg/kg range led to significant antitumor effects, with ER-086526 showing greater efficacy in all of the models. Thus, in the MDA-MB-435 model (Fig. 7, A and B), treatment with 0.25–1.0 mg/kg ER-076349 led to 60–70% inhibition at day 42, whereas ER-086526 at the same levels led to >95% inhibition, including actual regression of measurable tumors present on day 14. For both compounds, tumor regrowth rates after cessation of dosing allowed efficacy comparisons with paclitaxel at its empirically determined MTD of 25 mg/kg. Again, ER-086526 showed superiority over ER-076349; regrowth in all of the ER-076349 groups preceded the paclitaxel group by about 5 weeks, whereas all of the doses of ER-086526 were either equally efficacious (0.25, 0.5 mg/kg) or superior (1 mg/kg) to 25 mg/kg paclitaxel. Interestingly, the therapeutic window of ER-086526 seemed unusually large for a cytotoxic drug; >95% tumor suppression occurred over the 4-fold dosing range of 0.25–1.0 mg/kg with no evidence of toxicity based on body weight losses or decreased water consumption (data not shown). In contrast, complete tumor suppression by paclitaxel with this dosing regimen is only seen between 15–25 mg/kg, with 10 mg/kg inducing only partial inhibition (data not shown); the therapeutic window for paclitaxel in this model is thus just 1.7-fold. The effects of 0.125 and 0.5 mg/kg ER-076349 and ER-086526 were tested in several human tumor xenograft models, including MDA-MB-435 breast cancer, COLO 205 colon cancer, LOX melanoma, and NIH:OVCAR-3 ovarian cancer (Fig. 7). In all of the cases, treatment with ER-076349 and ER-086526 in the 0.05–1 mg/kg range led to significant antitumor effects, with ER-086526 showing greater efficacy in all of the models. Thus, in the MDA-MB-435 model (Fig. 7, A and B), treatment with 0.25–1.0 mg/kg ER-076349 led to 60–70% inhibition at day 42, whereas ER-086526 at the same levels led to >95% inhibition, including actual regression of measurable tumors present on day 14. For both compounds, tumor regrowth rates after cessation of dosing allowed efficacy comparisons with paclitaxel at its empirically determined MTD of 25 mg/kg. Again, ER-086526 showed superiority over ER-076349; regrowth in all of the ER-076349 groups preceded the paclitaxel group by about 5 weeks, whereas all of the doses of ER-086526 were either equally efficacious (0.25, 0.5 mg/kg) or superior (1 mg/kg) to 25 mg/kg paclitaxel. Interestingly, the therapeutic window of ER-086526 seemed unusually large for a cytotoxic drug; >95% tumor suppression occurred over the 4-fold dosing range of 0.25–1.0 mg/kg with no evidence of toxicity based on body weight losses or decreased water consumption (data not shown). In contrast, complete tumor suppression by paclitaxel with this dosing regimen is only seen between 15–25 mg/kg, with 10 mg/kg inducing only partial inhibition (data not shown); the therapeutic window for paclitaxel in this model is thus just 1.7-fold. The effects of 0.125 and 0.5 mg/kg ER-076349 and ER-086526 were tested in several human tumor xenograft models, including MDA-MB-435 breast cancer, COLO 205 colon cancer, LOX melanoma, and NIH:OVCAR-3 ovarian cancer (Fig. 7). In all of the cases, treatment with ER-076349 and ER-086526 in the 0.05–1 mg/kg range led to significant antitumor effects, with ER-086526 showing greater efficacy in all of the models. Thus, in the MDA-MB-435 model (Fig. 7, A and B), treatment with 0.25–1.0 mg/kg ER-076349 led to 60–70% inhibition at day 42, whereas ER-086526 at the same levels led to >95% inhibition, including actual regression of measurable tumors present on day 14. For both compounds, tumor regrowth rates after cessation of dosing allowed efficacy comparisons with paclitaxel at its empirically determined MTD of 25 mg/kg. Again, ER-086526 showed superiority over ER-076349; regrowth in all of the ER-076349 groups preceded the paclitaxel group by about 5 weeks, whereas all of the doses of ER-086526 were either equally efficacious (0.25, 0.5 mg/kg) or superior (1 mg/kg) to 25 mg/kg paclitaxel. Interestingly, the therapeutic window of ER-086526 seemed unusually large for a cytotoxic drug; >95% tumor suppression occurred over the 4-fold dosing range of 0.25–1.0 mg/kg with no evidence of toxicity based on body weight losses or decreased water consumption (data not shown). In contrast, complete tumor suppression by paclitaxel with this dosing regimen is only seen between 15–25 mg/kg, with 10 mg/kg inducing only partial inhibition (data not shown); the therapeutic window for paclitaxel in this model is thus just 1.7-fold.
In contrast, doubling the paclitaxel dose to 25 mg/kg in this model is lethal, whereas halving it to 6.25 mg/kg leads to only minor inhibition (data not shown); the therapeutic window for paclitaxel with this dosing regimen is thus <2-fold.

Inhibition of LOX tumor growth by ER-086526 was similar to that with ER-076349 but with about 2-fold greater potency. Thus, 0.05 mg/kg ER-086526 inhibited tumor growth by 78% at day 17, with higher doses of 0.1, 0.25, and 0.5 mg/kg ER-086526 leading to complete tumor suppression. Again, this represents an unusually wide 5-fold therapeutic window. Tumor regrowth rates in the 0.5 mg/kg ER-086526 group were delayed significantly beyond the 12.5 mg/kg paclitaxel group; regrowth in the 0.25 mg/kg ER-086526 group was slightly delayed relative to paclitaxel. Significantly, one and three mice in the 0.25 and 0.5 mg/kg ER-086526 groups (n = 10), respectively, became tumor-free by day 17; all of the mice had measurable tumors on day 10. These cured mice remained completely tumor-free for an additional 7 months. Other than a 2% decrease in body weights in the 0.5 mg/kg ER-086526 group during the first five daily injections, there were no other indications of toxicity in any group with either compound, as assessed by body weights or water consumption (data not shown). The slight decrease in body weights in the latter group resolved itself before the beginning of the second cycle of five daily injections and was not observed again.

Finally, effects of 0.125–1.0 mg/kg ER-076349 and ER-086526 on NIH:OVCAR-3 ovarian cancer xenograft growth were examined (Fig. 7, G and H). During actual dosing periods, both compounds inhibited tumor growth to about the same degree, with 0.5 and 1 mg/kg leading to inhibition during dosing equivalent to paclitaxel at its empirically determined MTD of 20 mg/kg. Two of six mice each in the 1 mg/kg ER-076349 and ER-086526 groups and six of six mice in the paclitaxel group became tumor-free by the end of dosing; these mice remained tumor-free through the end of the experiment (day 89). After cessation of dosing, increased efficacy of ER-086526 over ER-076349 became evident in the 0.5 and 1.0 mg/kg groups; no dose of ER-076349 achieved the long-term complete regrowth suppression seen with paclitaxel, whereas 1 mg/kg ER-086526 achieved complete suppression out to day 89, similar to paclitaxel. Significant body weight losses during dosing were only seen in the 20 mg/kg paclitaxel and 1 mg/kg ER-086526 groups (12 and 18% decreases, respectively); body weights in these groups recovered within about 10 days after cessation of dosing (data not shown).

DISCUSSION

In the 15 years since its discovery, the extraordinary in vitro and in vivo anticancer potency of halichondrin B (2, 10) has led to considerable interest in developing this agent as a new anticancer drug. Indeed, work on this compound by the NCI was ongoing as late as 1999 (28). Nevertheless, the extremely limited supply of halichondrin B from marine sources has all but eliminated the possibility that this promising agent might become a viable drug candidate. Fortunately, the complete synthesis of halichondrin B (11) and the subsequent discovery that its biological activity resides in its macrocyclic lactone C1-C38 moiety9 (see also Refs. 12–14) created the possibility of developing structurally simpler, fully synthetic analogues that retain the remarkable activity of halichondrin B. The resultant synthesis and evaluation of more than 180 macrocyclic analogues have culminated in the identification of ER-076349 and ER-086526.

ER-076349 and ER-086526 differ only in their C35 alcohol and amine substituents, respectively. Both showed sub-MIC in vitro growth inhibition against a variety of human cancer cell lines (Table 1), with the alcohol ER-076349 being about 2-fold more potent than the amine ER-086526 in most lines. Both were roughly 5–10-fold more potent in the COLO 205 colon cancer xenograft model (Fig. 7, C and D). As in the MDA-MB-435 study, ER-086526 showed superior efficacy, with frank regression and long-term suppression of tumor regrowth at 0.5 mg/kg and small but measurable decreases in growth rates at 0.125 mg/kg. Suppression with 0.5 mg/kg ER-086526 was superior to that of paclitaxel at its empirically determined MTD of 20 mg/kg, both in rapidity of regression onset and in duration of regrowth suppression. In contrast, inhibition by ER-076349 was less pronounced at these dosing levels; no measurable effects were seen at 0.125 mg/kg, and inhibition at 0.5 mg/kg was less than that with 20 mg/kg paclitaxel. As in the MDA-MB-435 study, there was no evidence of toxicity based on body weights or water consumption (data not shown).

Evaluation of 0.1–1.0 mg/kg ER-076349 and 0.05–0.5 mg/kg ER-086526 in the LOX melanoma model also showed potent antitumor effects of both compounds (Fig. 7, E and F). With ER-076349, all of the doses led to >90% tumor suppression by day 17, three days after cessation of dosing. The three top doses, 0.25, 0.5, and 1 mg/kg, led to virtually complete tumor suppression up to day 17, with inhibition of regrowth persisting well beyond that seen with 12.5 mg/kg paclitaxel, its empirically determined MTD with this regimen. Again, complete tumor suppression by 0.25–1.0 mg/kg ER-076349 in the LOX model represents an unusually wide 4-fold therapeutic window.
Fig. 7. Inhibition of human tumor xenograft growth in vivo by ER-076349, ER-086526, and paclitaxel. Nude mice bearing MDA-MB-435 breast cancer (A, B), COLO 205 colon cancer (C, D), LOX melanoma (E, F), and NIH: OVCAR-3 ovarian cancer (G, H) xenografts were treated with the indicated concentrations of ER-076349 (A, C, E, G), ER-086526 (B, D, F, H), and paclitaxel (all of the panels) as described in “Materials and Methods.” In the MDA-MB-435, COLO 205, and NIH: OVCAR-3 models, ER-076349 and ER-086526 studies were run as side-by-side comparisons but are plotted separately for ease of viewing; the same control and paclitaxel groups are thus replicated in each pair of graphs. The two LOX melanoma studies were run separately, each with its own control and paclitaxel groups. Periods of dosing are indicated by gray bars underlying the X axes; specific details of dosing schedules and routes of administration are presented in “Materials and Methods.” Plotted mean tumor volumes occurring after the onset of any complete remissions (see text) represent averages of only those animals continuing to bear measurable tumors.
than vinblastine and paclitaxel, two tubulin-based antimitotic agents run as internal standards in each experiment.

Direct comparisons of ER-076349 and ER-086526 with halichondrin B were not performed in the current study because of the lack of availability of either synthetic or natural halichondrin B. However, the low-to-sub-nM in vitro potencies measured for the two macrocyclic analogues were similar to historical halichondrin B data from our laboratory and others, indicating that the in vitro potency of halichondrin B had been retained during structural simplification. Thus, in an early study with synthetic halichondrin B, we measured a mean IC$_{50}$ of 0.4 nM against 17 human tumor cell lines representing eight different cancer types (24), whereas other laboratories using natural halichondrin B have reported IC$_{50}$ values of 0.08 nM, 0.3 nM, and 5 nM against murine B16 melanoma, murine L1210 leukemia, and PK1 normal kangaroo rat kidney cells, respectively (2, 6, 8). Moreover, performance of ER-076349 and ER-086526 in the 60-cell line screen of NCI was essentially identical to that of natural halichondrin B$^5$ (data not shown). We conclude that virtually all of the growth inhibitory potency was retained during structural simplification, including removing the entire C36-C54 polyether “left half” of halichondrin B and replacing the macrocyclic lactone ester of the latter with a nonhydrolyzable ketone bridge.

Our results indicate that the growth inhibitory mechanisms of ER-076349 and ER-086526 are probably the same as those of halichondrin B. Thus, ER-076349 and ER-086526 induce G2-M arrest and disrupt mitotic spindles, similar to previous results with synthetic halichondrin B (24) and consistent with the tubulin-based antimitotic mechanism of the latter (6–9). Moreover, our studies with the biotinylated macrocyclic lactone analogue ER-040798 showed direct binding to tubulin under affinity chromatography conditions (Fig. 5). Although ER-040798 was prepared before discovery of the macrocyclic ketone series, direct tubulin binding by ER-040798 is probably representative of this entire class of drugs because simplification from halichondrin B to the macrocyclic ketones involved numerous incremental modifications made without significant changes in growth inhibitory potency, G2-M arrest characteristics, or mitotic spindle disruption. Indeed, ER-076349 and ER-086526 directly inhibit tubulin polymerization in vitro with IC$_{50}$ values (5.7 μM and 6.9 μM, respectively) almost identical to the 7.2 μM value reported for halichondrin B (6). We conclude that the anticancer activities of ER-076349 and ER-086526 derive from a tubulin-depolymerizing antimitotic mechanism similar or identical to that reported for halichondrin B.

ER-076349 and ER-086526 showed significant in vivo anticancer efficacy at doses well below 1 mg/kg in several human tumor xenograft models. Interestingly, despite somewhat lower in vitro potency in cell growth and tubulin polymerization studies, ER-086526 showed greater in vivo efficacy, particularly in the MDA-MB-435 breast and COLO 205 colon cancer models. One explanation for this might be that ER-076349 is metabolized in vivo more readily than ER-086526; pharmacokinetic studies are under way to investigate this possibility. Another explanation might relate to our finding that mitotic blocks induced by ER-086526 are much less reversible after drug washout than those induced by ER-076349 (data not shown). This could result in greater in vivo tumor cell killing under the intermittent dosing schedules used in our studies. This observation, which correlates with potential for in vivo efficacy within the halichondrin B macrocyclic analogue series, will be described in a separate manuscript.$^6$

In all of the four in vivo models, ER-076349 and ER-086526 were
directly to tubulin polymerization studies, ER-086526 significantly wider in vivo therapeutic windows. For paclitaxel, experimentally determined therapeutic windows were relatively small, 1.7 in the MDA-MB-435 model and <2.0 in the LOX model. In marked contrast, therapeutic windows for the macrocyclic ketone analogues were much wider. In the MDA-MB-435 model, ER-086526 showed a 4-fold therapeutic window, whereas in the LOX model, therapeutic windows of 4- and 5-fold were observed for ER-076349 and ER-086526, respectively. We speculate that the wide therapeutic windows seen with ER-076349 and ER-086526 contribute to their substantial in vivo efficacy, in that the ability to increase doses 4–5-fold above fully tumor-suppressive doses probably leads to more complete eradication of residual tumor cells. This might explain the superior in vivo efficacy of ER-086526 over paclitaxel MTD dosing in three of the four models tested and the existence of complete cures in the LOX model by ER-086526. The reasons for the unusually wide therapeutic windows of ER-076349 and ER-086526 are not currently known.

In summary, we have demonstrated highly potent in vitro and in vivo anticancer activities of two fully synthetic, macrocyclic ketone analogues of halichondrin B, ER-076349, and ER-086526. These agents exert their anticancer effects by mechanisms currently indistinguishable from the microtubule-stabilizing effects of halichondrin B. An unexpected but exciting finding was the existence of unusually wide in vivo therapeutic windows, which may contribute to the remarkable in vivo efficacy of these new agents. Our encouraging early results with ER-076349 and ER-086526 strongly support their continued development as novel anticancer agents for human use.

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In Vitro and In Vivo Anticancer Activities of Synthetic Macrocyclic Ketone Analogues of Halichondrin B

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