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The microtubule binding drug laulimalide inhibits VEGF-induced human endothelial cell migration, and is synergistic when combined with Taxotere (docetaxel)

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Running Title: Laulimalide inhibits endothelial cell signaling and migration

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Abbreviations used:
ECM, extracellular matrix
FAK, focal adhesion kinase
HUVEC, human umbilical vein endothelial cells
VEGF, vascular endothelial growth factor
VEGFR, vascular endothelial growth factor receptor

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ABSTRACT

Laulimalide, a natural product from marine sponges, is a microtubule-stabilizing agent that binds to tubulin at a site distinct from that of the taxoids. In the present study, we found that laulimalide inhibited human umbilical vein endothelial cell (HUVEC) tubule formation and VEGF-induced HUVEC migration, key components of the angiogenic process. These occurred at concentrations substantially lower than that which inhibited HUVEC proliferation. When combined, laulimalide and Taxotere synergistically inhibited migration and tubule formation, but their combined effect on proliferation was antagonistic. Possible mechanism(s) by which laulimalide inhibited VEGF-induced HUVEC migration were explored. Similar to Taxotere, laulimalide had no effect on the VEGF-induced tyrosine phosphorylation of the VEGF receptor Flk-1/KDR (VEGFR-2). Also similar to Taxotere, low concentrations of laulimalide substantially blocked subsequent VEGFR-2 downstream events, including the phosphorylation of the Tyr^{397} and Tyr^{407} residues of focal adhesion kinase (FAK), the association of VEGFR-2 with FAK and Hsp90, and the Tyr^{31} phosphorylation of paxillin. Laulimalide inhibited integrin activation, however, compared to Taxotere, it had a weaker inhibitory effect on the VEGF-induced association of VEGFR-2 with the $\alpha_5\beta_1$ integrin. Compared to Taxotere, laulimalide more potently caused a reduction in the constitutive levels (i.e. in the absence of VEGF) of phosphorylated paxillin, and more potently inhibited the association of Rho A with the $\alpha_5\beta_1$ integrin. In conclusion, while both Taxotere and laulimalide inhibited integrin-associated signaling pathways which mediated VEGF-induced cell migration, their actions on the signaling cascade appeared not to be identical. These complementary actions could account for their synergistic effects on HUVECs.

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Microtubules are major components of the cytoskeleton and are necessary for the directed migration of endothelial and other cells (Liao et al., 1995). The microtubule network in interphase cells is a dynamic polarized structure, and during migration, the fast growing plus ends of the microtubules are targeted to and captured by the forming focal adhesions at the cell surface, while the stable minus ends are localized to the microtubule organizing center (Kaverina et al., 1998). In addition to their effects on tumor cell proliferation and apoptosis, agents that target the microtubule cytoskeleton can interfere with endothelial cell migration, and have been shown to be highly potent inhibitors of angiogenesis (Goldman, 1971; Zakhireh and Malech, 1980; Liao et al., 1995; Belotti et al., 1996; Hotchkiss et al., 2002). The microtubule-binding drugs have a number of cellular actions which could contribute to their inhibitory effects on cell migration, including impairment of the repositioning of the microtubule organizing center and interfering with the interaction of the microtubules with the developing focal adhesions (Hotchkiss et al., 2002). There is direct evidence that the cycle of microtubule polymerization and depolymerization can regulate the activity of the Rho GTPases RhoA and Rac1, and microtubule inhibitors could interfere with the functions of these regulatory proteins (Wittmann and Waterman-Storer, 2001. The microtubule system is also involved in intracellular protein trafficking and vesicle transport, and agents that inhibit microtubule plasticity could alter the formation of lamellipodia and the development of cell polarization (Hamm-Alvarez et al., 1994). Finally, the microtubule cytoskeleton has been shown to participate in the control of integrin clustering and avidity, thus providing a potential mechanism by which microtubule-binding agents could affect early migration-signaling events (Zhou et al., 2001).

Endothelial cell migration plays an essential role in angiogenesis and it is mediated by focal adhesions, structures which connect the extracellular matrix (ECM) with the plasma membrane and the underlying actin cytoskeletal network (Stokes and Lauffenburger, 1991). ECM proteins serve as ligands for cell surface integrins, and the attachment of cells to the ECM results in the clustering of integrin receptors and initiates the recruitment of additional

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cytoplasmic proteins to the focal adhesion complex, including structural and catalytically-active signaling proteins. Focal adhesions are dynamic structures, and their formation and breakdown are regulated by many different extracellular stimuli, including VEGF (Abedi and Zachary, 1997; Rousseau et al., 1997). Formation of these complexes has been shown to require the activity of the small Rho GTP-binding proteins (Soga et al., 2001; Zeng et al., 2002). VEGF is a multifunctional cytokine that stimulates endothelial cell proliferation and migration, increases microvascular permeability, and is required for tumorigenesis as well as angiogenesis (Ferrara, 2002; Millauer et al., 1993). The biological effects of VEGF on endothelial cells are mediated through the activation of its receptor tyrosine kinases, including Flt-1 (VEGFR-1) and Flk-1/KDR (VEGFR-2), with the latter responsible for VEGF-induced endothelial cell migration (Millauer et al., 1993; Waltenberger et al., 1994). Signaling events which follow VEGFR-2 phosphorylation include the activation of integrins, the phosphorylation of proteins involved in cell migration signaling including focal adhesion kinase (FAK), and the recruitment of growth factor receptors, integrins and downstream effectors into newly formed focal adhesions (Le Boeuf et al., 2004; Avraham et al., 2003; Masson-Gadais et al., 2003).

Microtubule-binding agents such as colchicine, nocodazole, vinblastine, Taxol and Taxotere have been shown to inhibit the migration of a range of cell types, including fibroblasts, monocytes, endothelial cells, and cell lines derived from lymphomas, melanomas and prostate carcinomas (Goldman, 1971; Zakhireh and Malech, 1980; Stearns and Wang, 1992; Liao et al., 1995; Hotchkiss et al., 2002). Laulimalide, a natural product that was first isolated from Pacific marine sponges, is a microtubule-stabilizing agent that binds to the tubulin polymer at a site that is distinct from that of the taxoids (Mooberry et al., 1999; Pryor et al., 2002). Laulimalide has been shown to be active against tumor cells that were resistant to the taxanes, both when resistance was due to overexpression of P-glycoprotein or when it was due to mutations in the β-tubulin gene (Mooberry et. al. 1999). In the present study, we found that laulimalide, similar to Taxotere, inhibited VEGF-induced endothelial cell migration at concentrations lower than those

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required to inhibit endothelial cell proliferation or stabilize microtubules. The purpose of the present study was to identify potential mechanism(s) by which laulimalide inhibited endothelial cell migration, to compare these actions to those of Taxotere, and to evaluate the nature of the interaction of laulimalide and Taxotere on endothelial cells.

MATERIALS AND METHODS

Reagents. Laulimalide was kindly provided by Scott Nelson and Billy Day, Univ. of Pittsburgh. Taxotere was from Aventis Pharmaceuticals (Parsippany, NJ).

Cell culture. Human umbilical vein endothelial cells (HUVEC) were purchased from GlycoTech, Inc. (Rockville, MD) by arrangement of the Angiogenesis Resources Branch of the Developmental Therapeutics Program (NCI). Culture medium consisted of MCDB131 with 2% FBS (GIBCO), 10 ng/ml EGF, 12 µg/ml ECGS, 1 µg/ml hydrocortisone, 10 units/ml heparin, 2 mM L-glutamine, 5 units/ml penicillin G, and 5 µg /ml streptomycin sulfate (all from Sigma).

Endothelial cell proliferation assay. 10^4 HUVEC were placed in each well of a 96 well plate. After allowing for attachment overnight, the laulimalide was added, and the number of cells after 6 days was determined by staining with sulforhodamine B (Sigma).

Assay of *in vitro* tubule formation. The spontaneous formation of capillary-like structures by HUVEC on a basement membrane matrix preparation, Matrigel (Becton Dickinson), was used to assess angiogenic potential. Twelve-well plates (Costar) were coated with Matrigel (10 mg/ml) according to the manufacturer's instructions, and HUVEC (2×10^5 cells/well) were seeded and incubated at 37°C for 60 min. Laulimalide was added, and i*n vitro* tubule formation was photographed after the cultures were incubated at 37°C for 24 h. Quantitation was done by counting the number of tubules having branch points at both ends in three low-power (10x) fields, a sensitive descriptor that has been shown to measure endothelial cell reorganization into a capillary-like network (Guidolin et. al, 2004).

Endothelial cell migration assay. The migration assay utilized a modified Boyden chamber. Confluent HUVEC monolayers were cultured with non-growth factor-containing media

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for 48 h before harvesting with Cell Dissociation Solution (Sigma). Harvested cells were suspended at 10^6/ml in M199 with 1% serum, and 10^5 cells seeded into transwell inserts (8 µm pore, Costar) precoated with 10 µg/ml fibronectin. Inserts containing HUVEC were placed into a 24-well plate containing 700 µl of M199 with 1% serum and incubated for 1 h at 37°C. HUVEC migration was stimulated by addition of the VEGF (10 ng/ml) to the lower well of the Boyden chamber. The effects of laulimalide, Taxotere, or the combination of the two compounds on endothelial migration was observed by their addition to the lower chamber. After 5 h, HUVEC were stained with 10 µM Cell Tracker Green (Molecular Probes, Eugene, OR) for 30 min at 37°C, and the upper membrane of the insert was swabbed to remove non-migrated cells. Inserts were washed with PBS, fixed in formaldehyde, and mounted on microscope slides. HUVEC migration was quantitated by counting the number of cells in five random fields (x100) per insert.

Some experiments used the CytoSelect 24-well cell migration assay kit (Cell Biolabs) to quantitate migration on the entire filter, rather than by counting individual cells. In this assay, after swabbing to remove non-migrated cells, the inserts were placed in cell staining solution for 10 min, and then shaken with 200 µl of extraction solution. HUVEC migration was quantitated by measuring the OD at 560 nm. Results from the two assays were comparable, and data presented are a combination of both.

Immunofluorescent staining. HUVEC were seeded on fibronectin (10 µg/ml)-covered chamber slides. After 4 h of incubation in serum-free M199 medium, the cells were treated with serial-diluted laulimalide for 1 h, and then stimulated with VEGF. The cells were fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature, and treated with blocking buffer (1% bovine serum albumin in PBS) for 1 h at RT. FAK (red) was visualized with a rabbit anti-FAK antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) and a Cy-3 conjugated anti-rabbit antibody. Tubulin (green) was visualized with an anti-α tubulin monoclonal antibody (Sigma) and a FITC-conjugated anti-mouse antibody. An engineered monovalent antibody which recognizes integrin $\alpha_v\beta_3$ in its high affinity state

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(WOW-1 Fab) (Pampori et. al., 1999; kindly provided by Dr. S. Shattil) or a mouse anti-integrin $\alpha_V\beta_3$ antibody (Santa Cruz Biotechnology) were used to assess the effects of VEGF and laulimalide on integrin activation.

Immunoprecipitation and western blots. Confluent HUVEC, seeded on fibronectin (10 µg/ml)-covered dishes and starved overnight in M199 with 1% FBS, were treated with serial dilutions of laulimalide or Taxotere for 1 h. Cells were stimulated with VEGF (10 ng/ml) for the indicated times. Monolayers were washed twice in PBS and treated with immunoprecipitation lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 1 mg/ml leupeptin). Cell suspensions were incubated on ice for 30 min and clarified by centrifugation. For immunoprecipitation, protein content was determined by the BCA method (Pierce), and 300 µg of total protein was incubated overnight at 4 °C with protein G-agarose beads coated with saturating amounts of antibodies to integrin $\alpha_V\beta_3$ (Santa Cruz Biotechnology), integrin $\alpha_5\beta_1$ (Chemicon International) or VEGFR-2 (Santa Cruz). The resulting immune complexes were recovered after centrifugation by boiling for 3 min in SDS-PAGE loading buffer. For direct immunoblotting of whole cell lysates, cells were lysed in a modified RIPA lysis buffer (Biosource International).

For immunoblotting, aliquots of whole cell lysates (30 µg) or isolated immunocomplexes were separated by SDS-PAGE under reducing conditions using 10% polyacrylamide gels. Proteins were transferred onto polyvinylidene difluoride membrane and analyzed by immunoblotting using antibodies against VEGFR-2, FAK, paxillin, integrin β_1 (all from Santa Cruz), RhoA, VEGFR-2 phosphorylated on Tyr^{951}, FAK phosphorylated on various tyrosine residues (Tyr^{397}, Tyr^{407}, Tyr^{576}, Tyr^{577}, and Tyr^{861}), and paxillin phosphorylated on Tyr^{31} (all from Biosource International). Antibodies against total FAK, paxillin and α-actin were used as control for loading when using whole cell lysates, whereas antibodies against integrins β_1 and VEGFR-2 were used to quantitate protein loading for the immunoprecipitation reactions.

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Drug combination effect analyses. A computerized version of the combined effects method was used to analyze the nature of the interaction of laulimalide and Taxotere on HUVEC migration, tubule formation and proliferation. Details of the method and its statistical basis have been previously described (Chou and Talalay, 1984; Bible and Kaufmann, 1997). For these experiments, the two agents were used either alone or together at several concentrations, in all cases at fixed molar ratios based on their IC_{50}s. The ratio used in the migration and tubule assays were 1:1 (Taxotere:laulimalide), while that in the proliferation assay was 1:6.25. Calcusyn software (Biosoft) was then used to calculate the combination index (CI) for each concentration tested, whereby CI values less than 1 indicated synergy, equal to 1 indicated additivity, and those greater than 1 indicating antagonism in the interaction of the drugs.

RESULTS

Laulimalide and Taxotere synergistically inhibited HUVEC migration and the formation of capillary-like structures at low concentrations that didn't inhibit HUVEC proliferation. Sprouting angiogenesis encompasses successive phases of microvessel formation, neovessel growth and neovessel stabilization (Vailhé et. al., 2001). Steps in the neovessel growth process include the migration of endothelial cells from the parent vessel toward an angiogenic factor, proliferation of endothelial cells behind the front of migration, and the organization of the endothelial cells into capillary-like structures. The creation of capillary-like structures *in vivo* and *in vitro* involves the remodeling and pruning (via apoptosis) of the endothelial cells, the formation of lumens, and the formation of loops by anastomoses (Vailhé et. al., 2001). These multi-step processes can be recapitulated with *in vitro* assays, which were used in this study to evaluate the effects of laulimalide and Taxotere. HUVEC migration in a modified Boyden chamber is a chemotactic model of migration representative of tumor-induced endothelial cell migration. Stimulation of HUVEC along a directional gradient of VEGF resulted in migration to the underside of the membrane, and this was inhibited in a concentration-

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dependent manner by laulimalide with an IC_{50} of about 0.01 nM, comparable to that of Taxotere (Fig. 1A).

When HUVEC are plated on a basement membrane matrix (Matrigel) in short term culture (24 hours), they undergo alignment into networks of tubules (Fig. 2A), a process that is dependent upon proteolytic degradation of the matrix, cell realignment, and apoptosis; directed cell migration and proliferation, however, are not involved in this process (Vailhé et. al., 2001). Laulimalide reduced HUVEC tubule formation in a concentration-dependent manner (Figs. 1B and 2), with a significant reduction ($p < 0.05$) observed at 0.01 nM and with an IC_{50} of approximately 0.8 nM (Fig. 1B). No tubules were formed at laulimalide concentrations of 5 nM or higher (not shown). An identical dose-response curve was seen with Taxotere. The drug concentrations which inhibited tubule formation were higher than those which inhibited HUVEC migration in the Boyden chamber assay, possibly due to differences in the cellular processes involved, as noted above, and also possibly due to the physical or chemical nature of the Matrigel matrix. The effect of laulimalide and Taxotere on HUVEC proliferation was also determined (Fig. 1C). In this instance, Taxotere was found to be more potent than laulimalide, with IC_{50}s of 1 nM and 4 nM, respectively. For laulimalide, this was 400-fold and 5-fold higher than the drug's effect on migration and tubule formation, respectively.

Laulimalide binds at a site on the tubulin polymer distinct from the taxoid binding site, and has a synergistic effect on microtubule polymerization when combined with taxol (Pryor et al., 2002; Gapud et. al., 2004). We therefore investigated the possibility that laulimalide and Taxotere, in combination, might also act synergistically to inhibit the processes involved in angiogenesis. We determined the effects of the two drugs when used alone and together, testing them at several equipotent concentrations (i.e. at their IC_{50}s and at constant ratios of their IC_{50}s). For each of these, the combination index (CI) was calculated and plotted versus the fraction affected (F_a) for the specific drug combination. As described by Chou and Talalay (1984), a CI value of 1.0 indicates drug additivity, $CI < 1$ synergy, and $CI > 1$ indicates

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antagonism. As seen in Figs. 1D and E, there was evidence for synergy in the cell migration and tubule assays, although in the case of migration, the degree of synergy diminished with increasing drug concentrations. The synergy was most dramatically seen in the tubule assay. When used individually at 0.1 nM, laulimalide and Taxotere had modest inhibitory effects on tubule formation (Figs. 2C and 2E, respectively). When used in combination at 0.1 nM, however, there was nearly complete inhibition of tubule formation (Fig. 2F). The effect of the combination was even greater than that of a 10-fold higher concentration of laulimalide alone (i.e. 1 nM; Fig. 2D). In contrast, there was no synergy observed for the effect of the two drugs on HUVEC cell proliferation at all concentrations tested, and in fact, the combinations appeared to be antagonistic (Fig. 1F).

Laulimalide inhibited VEGF-induced cell polarization, focal adhesion formation, radial microtubule growth, and integrin $\alpha_v\beta_3$ activation. Cell migration is a coordinated process consisting of adhesion at the leading edge and detachment at the rear of the cell. Adhesion to ECM involves structures heterogeneous with respect to size, composition, and orientation to actin filaments, the largest and tightest structures of which are focal adhesions. Focal adhesions link the actin cytoskeleton to the ECM by integrin receptor complexes. These processes can be readily visualized in endothelial cells as VEGF and other chemotactic factors initiate a series of morphologic changes. HUVEC were plated on fibronectin-coated coverslips, stimulated with VEGF in the absence or presence of laulimalide for one hour, and then fixed and labeled for both tubulin (green) and FAK (red) (Fig. 3). Control endothelial cells predominantly had a typical polygonal shape with a relatively uniform distribution of FAK within the cytoplasm and on the cell surface, and a poorly visible microtubule skeleton. As can be seen in Fig. 3, VEGF induced cell spreading, flattening and polarization, and the formation of membrane protrusions, precursors to nascent lamellipodia. The size and number of FAK-containing focal adhesions increased in VEGF-treated cells, and FAK and the focal contacts appeared to redistribute to the protruding edges of the cell membranes. The microtubules became more

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visible, and grew radially toward the expanding regions of the cell periphery. All of the VEGF-induced changes were inhibited in a concentration-dependent manner by laulimalide, with substantial inhibition seen at 0.1 nM and complete inhibition at 1 nM. It was only at the highest concentration tested, 10 nM, at which both HUVEC migration and proliferation were inhibited, that the cells acquired the rounded morphology and condensed microtubules typically seen at cytotoxic concentrations.

Integrins mediate cell adhesion and signaling, and therefore play a critical role in cell migration. Their primary function is ligand binding, and this is regulated in part by conformational changes in the $\alpha\beta$ heterodimers that increase integrin affinity for their ECM ligands and thereby cause integrin activation. Previous work showed that VEGF-stimulated HUVEC migration is mediated through two distinct integrins, $\alpha_V\beta_3$ and $\alpha_5\beta_1$, and VEGF stimulates the recruitment of activated, high affinity integrins to the leading edge of migrating endothelial cells (Byzova et al., 2000; Hotchkiss et al., 2003). To investigate whether laulimalide was capable of suppressing integrin activation, we used WOW-1, an engineered, monovalent, ligand-mimetic Fab fragment that reacts selectively with $\alpha_V\beta_3$ when it is activated and in a high affinity state (Pampori et al., 1999). As illustrated in Fig. 3, WOW-1 staining in control cells exhibited a nuclear distribution, and this most likely was due to nonspecific interactions. VEGF treatment resulted in $\alpha_V\beta_3$ activation, as judged by the more intense WOW-1 staining including around the cell periphery. Incubation of the cells with laulimalide blocked the VEGF-induced integrin $\alpha_V\beta_3$ activation, and as before, partial inhibition was observed at 0.1 nM, and complete inhibition at 1 nM Taxotere. In contrast, neither VEGF nor laulimalide had any effect on the levels or distribution of total $\alpha_V\beta_3$ integrins (Fig. 3).

Laulimalide had no effect on VEGF-induced VEGFR-2 phosphorylation, but did inhibit FAK and paxillin phosphorylation. The cellular actions of VEGF are triggered by its binding to its cognate receptors, with VEGFR-2 playing the predominant role in endothelial cell migration. Thus tyrosine phosphorylation of VEGFR-2 represents one of the first events in the

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signaling cascade whereby VEGF induces cell migration. To determine if VEGFR-2 activation was affected by laulimalide, HUVEC were seeded on fibronectin, starved in serum-free medium overnight, treated with laulimalide for 1 h, and stimulated with VEGF (10 ng/ml) for 5 min. Using an antibody that was specific for Tyr^{951}-phosphorylated VEGFR-2, we found that laulimalide had no effect on VEGF-induced receptor phosphorylation, when compared to cells stimulated with VEGF alone (Fig. 4A). Identical results were obtained when total VEGFR-2 was immunoprecipitated and then probed with an anti-phosphotyrosine antibody (not shown).

Integrins have short cytoplasmic tails that lack catalytic activity, and thus their ability to transmit outside-in cell signaling is mediated by cytoplasmic proteins that localize to sites of clustered and activated integrins. One such protein is FAK, a 125 kDa cytoplasmic tyrosine kinase that is localized to focal adhesions, where it acts to integrate growth factor and integrin signals (Hanks et al., 1992). FAK becomes phosphorylated at six different tyrosine residues after the engagement of integrins with ECM proteins, and these phosphorylations are important for cell migration (Sieg et al., 2000). Since laulimalide inhibited VEGF-induced HUVEC migration and focal adhesion formations, we next examined its effect on FAK phosphorylation, using phosphorylation site-specific antibodies. Phosphorylation of FAK on Tyr^{397} (the autophosphorylation site) occurred rapidly upon VEGF stimulation, reaching a maximum at five minutes and declining to basal level by 30 minutes (not shown). There were smaller increases in VEGF-induced FAK phosphorylation at tyrosine residues 407, 576, 577 and 861 (Fig. 4B). Laulimalide most prominently inhibited phosphorylation of FAK on Tyr^{397}, with a substantial decrease observed at 0.01 nM and a reduction to near baseline levels at 0.1 nM laulimalide (Fig. 4B). It also blocked VEGF-induced phosphorylation at Tyr^{407}, although this site seemed to be somewhat less sensitive to inhibition by laulimalide. Laulimalide had no effect on the more modest increases in FAK phosphorylation at tyrosines 576 and 861.

FAK is required for the downstream signaling from VEGFR-2 in endothelial cells, and its actions are mediated by the heat shock protein Hsp90, which acts as a bridging protein to

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stabilize the physical association of FAK and VEGFR-2 (Le Boeuf et al., 2004). The interaction of Hsp90 and FAK with VEGFR-2 was lost in cells treated with laulimalide (Fig. 4C), and this action of the drug likely prevents signal transduction from the VEGF receptor and could account for its inhibitory effects. FAK also plays a key role in the dynamic reorganization of the cytoskeletal network that precedes cell migration. Its phosphorylation provides sites for the interaction with a number of focal adhesion-associated proteins, and we next examined one of these, paxillin. Paxillin is phosphorylated in VEGF-treated cells on Tyr^{31}, and this occurred maximally at 30 minutes after VEGF addition, indicating, as expected, that it occurred downstream and likely as a consequence of FAK phosphorylation (not shown). The phosphorylation of paxillin is dependent upon FAK phosphorylation, and since the latter was inhibited by laulimalide, we expected that paxillin phosphorylation would likewise be inhibited. This can be seen in Fig. 4D, where the increase in paxillin phosphorylation was blocked by low concentrations of laulimalide.

Laulimalide suppressed the basal tyrosine phosphorylation level of paxillin. There was a low level of tyrosine phosphorylation of both FAK and paxillin observed under control conditions (in the absence of VEGF), and our investigations of the effect of laulimalide suggested that the drug could be also reducing this basal level, in addition to blocking the VEGF-induced effect. Evaluation of this possible drug action could be done using a longer exposure of the immunoblots. As shown in Fig. 5, laulimalide prominently inhibited the basal phosphorylation of paxillin on Tyr^{31}, with a substantial decrease observed at 0.01 nM. Taxotere showed a substantially weaker effect, with only a modest decrease observed at 1 nM (Fig. 5). Neither laulimalide nor Taxotere affected the basal tyrosine phosphorylation level of the autophosphorylation site of FAK (Tyr^{397}) (not shown).

Association of integrin $\alpha_5\beta_1$ with signaling pathways: Taxotere but not laulimalide blocked association with VEGFR-2, while laulimalide but not Taxotere blocked association with RhoA. Recent studies have shown that the mechanisms by which growth

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factor receptors and integrins regulate cellular functions are not only coordinated, but also are interdependent (Byzova et al., 2000; Soldi et al., 1999; Sieg et al., 2000). As such interactions have been shown to mediate the actions of VEGF, the association of VEGFR-2 with integrins likely represents one of the immediate down-stream events of the occupancy, phosphorylation and activation of VEGFR-2. We determined whether laulimalide and Taxotere influenced the association of VEGFR-2 with integrins by co-immunoprecipitation of lysates from laulimalide, Taxotere and VEGF-treated cells using monoclonal antibodies against integrin $\alpha_V\beta_3$ and $\alpha_5\beta_1$. The resulting complexes were subjected to SDS-PAGE and were probed for the presence of VEGFR-2 (Fig. 6A). In agreement with a previous study, VEGFR-2 was found to coimmunoprecipitate with integrin $\alpha_5\beta_1$, and this association was increased upon VEGF treatment of HUVEC that were plated on fibronectin (Wijelath et al., 2002). In further agreement with this study, we were unable to detect VEGFR-2/integrin $\alpha_V\beta_3$ complexes in control or VEGF-treated HUVEC, although these complexes have been observed in cells plated on different matrix substrates (Soldi et al., 1999; Wijelath et al., 2002). The VEGF-induced association of VEGFR-2 with $\alpha_5\beta_1$ was reduced in a concentration-dependent manner in Taxotere-treated cells, while there was no change in laulimalide-treated cells.

The Rho family of small GTPases is involved in the regulation of several components of cell migration, including the development of cell polarization, the assembly of focal adhesions, the formation of directional cell protrusions, and the rapid reorganization of actin filaments. They also regulate the local stabilization of microtubules at the leading edge of migrating cells in a process that requires integrin-mediated activation of FAK (Palazzo et. al., 2004). VEGF induced the activation of RhoA and its recruitment to the cell membrane of endothelial cells, and RhoA has been shown to be required for VEGFR-2-mediated endothelial cell migration (Zeng et. al., 2002; van Nieuw Amerongen et. al., 2003). We examined the effect of laulimalide and Taxotere on the association of RhoA with integrins, and as shown in Fig. 6B, found that laulimalide strongly blocked the association of RhoA with integrin $\alpha_5\beta_1$, an effect which was

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apparent at 0.01 nM of laulimalide. Total RhoA levels in the cell lysates were not affected (Fig. 6C). As in its effects on paxillin, the actions of laulimalide on RhoA could be distinguished from those of Taxotere, which only weakly reduced the RhoA-integrin association.

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DISCUSSION

Angiogenesis, the process of sprouting of capillaries from preexisting blood vessels, plays an important role in the process of tumor growth and metastasis. Endothelial cell activation, migration, proliferation, and differentiation are major cellular events in this process. Microtubule-interfering agents were among the first of the cytotoxic chemotherapeutic drugs to be reported to have an anti-angiogenic effect, and most members of this class of agents have been shown to have this activity (Goldman, 1971; Zakhireh and Malech, 1980; Liao et al., 1995; Belotti et al., 1996; Hotchkiss et al., 2002). Given the multiple roles that microtubules play in cell migration, there are potentially several sites and mechanisms by which microtubule-disrupting compounds could be acting to block cell motility. Some of the earlier studies, in which the high concentrations of these agents used caused near complete microtubule breakdown, are likely not relevant to the effects of microtubule-stabilizing drugs observed in this report or to the concentrations of these drugs which occur clinically. Rather, we have found inhibition of endothelial cell migration *in vitro* at low concentrations that did not affect gross microtubule structure. These observations suggested that the drugs are either having more subtle effects on microtubule plasticity and dynamics, or that they were acting at sites distinct from, or in addition to, their effects on microtubules.

A mechanism-based screening of marine sponges led to the identification of a macrocyclic lactone, laulimalide, with microtubule stabilizing properties remarkably similar to those of the taxanes (Moobery et. al., 1999; Gapud et. al., 2004). Consistent with this observation, in this report we found that laulimalide inhibited human endothelial cell migration and tubule formation with a potency equal to that of Taxotere. Despite these similarities, previous studies found that laulimalide does not bind to the taxoid site on β-tubulin, and when used at suboptimal concentrations, acted synergistically with taxol in inducing tubulin assembly in cell-free systems (Pryor et. al., 2002; Gapud et.al, 2004). Computational analyses suggested that while laulimalide could bind to the taxoid site, its preferred binding site on the αβ-tubulin

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dimer was over a mobile region of α-tubulin, the B9-B10 loop extension, a site distinct from that which binds the taxanes and colchicine (Pineda et al., 2004). This explains why laulimalide was active against tumor cells made resistant to the taxanes by mutations in the β-tubulin gene, since the amino acids involved in the mutations were not predicted to interact with laulimalide, according to this model (Pineda et. al., 2004). Laulimalide was also active in cells that were resistant to the taxanes due to overexpression of P-glycoprotein, suggesting that there were significant differences in the nature of the interaction of laulimalide with P-glycoprotein as well, when compared to the taxanes. Our studies of the molecular actions of laulimalide suggested that while it might share, with the taxanes, similar target protein(s) for the inhibition of cell migration, it also interacts with these proteins in a manner that differs from that of the taxanes. It is presumed that it is these differences that contribute to the synergistic inhibition of migration and tubule formation by the combination of laulimalide and Taxotere found in this study.

Inhibition of HUVEC proliferation by laulimalide occurred with an IC_{50} of about 4 nM, which was similar to its IC_{50} for the inhibition of proliferation of several tumor cell lines (Mooberry et al., 1999). More interesting was the observation that tubule formation and VEGF-induced migration of HUVEC was shown to be reduced by laulimalide at low sub-nanomolar concentrations, suggesting that laulimalide and related compounds could have therapeutically-relevant anti-angiogenic actions. In addition to being a potent inhibitor of VEGF's effects, these actions will likely apply to effects on endothelial cells that are mediated by other angiogenic factors, as laulimalide inhibited paxillin phosphorylation and tubule formation in the absence of VEGF. The data obtained demonstrated that laulimalide inhibited signal transduction pathways involved in the initiation of endothelial cell migration. Inhibition likely occurred at a point subsequent to the binding of VEGF and the activation of the VEGF receptor, as laulimalide did not affect VERGFR-2 tyrosine phosphorylation. Given the complexity and inter-connected nature of these downstream signaling events, it is not possible to precisely pinpoint the site of action of laulimalide, although the effect of laulimalide to block the association of VEGFR-2 with

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Hsp90 and FAK is an early event and thus is a likely site of action (Rousseau et. al. 2000; LeBoeuf et. al., 2004).

Our data demonstrated that laulimalide, similar to Taxotere, suppressed the activation of integrins upon treatment with VEGF. Integrins, which are specific protein receptors for ECM, are activated as a result of an increase in their binding affinity and by their clustering at the cell surface (avidity), and the inhibition of their activation by laulimalide has consequences for two related pathways (Parsons et. al., 2000). Data suggest that the VEGF receptor forms complexes with the integrins in a manner which promotes reciprocal cross-talk and cooperative actions of the VEGF- and ECM-receptors to enhance their respective cellular responses (Soldi et. al., 1999; Byzova et. al., 2000; Wijelath et. al., 2002). In contrast to the effect of Taxotere, however, laulimalide did not block the association of VEGFR-2 with the integrins, suggesting that this cross-talk was not a likely site of laulimalide action. Activation of integrins also leads to the subsequent activation, via protein phosphorylation, of FAK and other focal adhesion-associated proteins, including paxillin, c-src, and $p130^{CAS}$ (Parsons et. al., 2000). FAK does not have intrinsic enzyme activity but rather serves a scaffold protein for the assembly of other regulatory molecules. Thus it was anticipated that the inhibition of VEGF-induced FAK activation by laulimalide would also lead to the inhibition of downstream, FAK-dependent proteins, and this was found to be the case for the FAK-binding protein, paxillin. That laulimalide also potently inhibited the basal level of paxillin activation was unexpected, however, and suggested that laulimalide could have an additional and independent action on paxillin or on a paxillin-related pathway. This action also provides an indication as to how laulimalide and related compounds might inhibit migration that is not initiated by VEGF or by the activation of other receptor tyrosine kinases.

Studies have implicated Rho-family members in the crosstalk between microtubules and actin that is required for the regulation of cell motility (Waterman-Storer et. al., 1999). During migration, microtubules emanate out from the center of the cell towards the leading edge, where

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their plus ends exhibit dynamic instability (random changes between periods of growth and shortening). The dynamic instability of the microtubules can activate signal-transduction cascades at the leading-edge of the cell, with depolymerization causing an increase in the level of GTP-bound RhoA, whereas the polymerization resulting in activation of the related GTPase Rac1 (Waterman-Storer et al., 1999). Ultimately there is a localized stabilization of the microtubules at the leading edge of cells undergoing migration, a process that has been shown to be FAK- and Rho-dependent (Palazzo et. al., 2004). Thus it is possible that by stabilizing microtubules in general or at specific sites within the cell, laulimalide and related agents impair the development of polarization in the cell, a process that is required for their directional motility. The ability of laulimalide to prevent integrin and FAK activation also would be anticipated to affect the Rho-mediated signal pathways, as Rho signaling is facilitated by FAK (Palazzo et. al., 2004). Alternatively, if one action of laulimalide is to block the activation of members of the Rho family via an effect on the microtubules, then this could explain the effect of laulimalide on FAK and the integrins. RhoA kinase activation has been reported to be involved in the activation of a tyrosine kinase that is responsible for phosphorylating Tyr^{407} of FAK (Hanks and Polte, 1997), while Rac has been shown to recruit high affinity integrins to the lamellipodia in endothelial cells (Kiosses et al., 2001) .

In summary, laulimalide and Taxotere had several effects on cell signaling that might contribute to their inhibitory effects on endothelial cell migration and tubule formation. These molecular effects likely represent novel sites of action of microtubule-binding drugs, distinct from their well-documented effects on mitosis, apoptosis, and cell proliferation. The synergistic inhibitory effects of laulimalide and Taxotere on endothelial cell migration may be due to their differing and/or complementary actions on signaling pathways participating in endothelial cell migration.

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Footnote

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Figure Legends

Figure 1: **Inhibition of endothelial cell migration, capillary-like tubule formation, and proliferation by laulimalide and Taxotere. (A)** For cell migration, HUVEC were starved in M199 media lacking growth supplements for 18 h, and then were harvested and seeded into transwell inserts (8 µm pore) pre-coated with 10 µg/ml fibronectin. Inserts containing HUVEC were placed in a 24-well plate containing M199 with 1% serum and incubated for 1 h at 37°C. Taxotere (○) or laulimalide (△) were added to the upper chamber for 1 h, and HUVEC migration was then initiated by the addition of VEGF (10 ng/ml) to the lower chamber. After an additional 5 h, migration of HUVEC to the underside of the transwell insert was quantitated as described in Methods. Data are from 3 experiments, and are expressed relative to the VEGF-alone control. **(B)** For tubule formation *in vitro,* HUVEC (2 x 10^5/well) were seeded onto Matrigel (10 mg/ml)-coated twelve-well plates and incubated at 37°C for 60 min. The indicated concentrations of Taxotere (○) or laulimalide (△) were added, and cells were incubated for an additional 24 h. Endothelial tubules were photographed and quantitated; only intact tubules with branch points at both ends were counted as positive. Data are from 3 experiments, and are expressed relative to the vehicle (DMSO)-treated control. **(C)** For cell proliferation, HUVEC cells were placed into a 96 well plate, and after allowing for cell attachment overnight, the indicated concentrations of Taxotere (○) or laulimalide (△) were added. The number of cells was determined after 3 days by staining with sulforhodamine B. Data are means of 4 experiments, and are expressed as a percent of vehicle-control. **(D, E and F)** Combination effects of laulimalide and Taxotere were determined for cell migration (D), tubule formation (E) and proliferation (F). Each assay was done as described in panels A-C, and data for the two drug, used individually and in combination, obtained. Drugs were combined at equipotent concentrations, which for migration and tubule formation was a molar ratio of 1:1, and for proliferation was a molar ratio of 1:6.25 (Taxotere:laulimalide). Drug effects are calculated as the combination index, and are expressed as a function of the fraction affected in the

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combination treatment. CI values less than 1 indicate that the drug combination had a synergistic interaction, values at or near 1 indicate the combination was additive, and values greater than 1 indicate an antagonistic interaction. Migration data are means of 8 experiments, tubule formation are the means of 2 experiments, and proliferation data are the means of 4 experiments.

Figure 2. Inhibition of endothelial cell capillary-like tubule formation by laulimalide and Taxotere. Tubule formation on Matrigel was done as described in the legend to figure 1B. Representative low-power magnification pictures are shown for HUVEC treated with vehicle control (A), laulimalide at concentrations of 0.01 nM (B), 0.1 nM (C) and 1 nM (D), Taxotere at 0.1 nM (E), or a combination of laulimalide and Taxotere, each at 0.1 nM (F).

Figure 3: Laulimalide inhibited VEGF-induced endothelial cell polarization, assembly of focal contacts into focal adhesions, and activation of integrin $\alpha_v\beta_3$. For the staining of focal adhesions, HUVEC were seeded on fibronectin, starved in M199 for 4 h, treated with laulimalide for 1 h at the indicated concentrations, and stimulated with VEGF (10 ng/ml) for 4 h. FAK (red) was visualized with a rabbit anti-FAK antibody (1:200; Santa Cruz) and a Cy-3 conjugated anti-rabbit antibody. Tubulin (green) was visualized with an anti-alpha tubulin monoclonal antibody (Sigma) and a FITC-conjugated anti-mouse antibody. For the staining of integrins, HUVEC were seeded on fibronectin and starved in DMEM for 6 h, treated with laulimalide for 1 h, and stimulated with VEGF (50 ng/ml) for 10 min. "Activated" integrin $\alpha_v\beta_3$ was determined by staining with an engineered antibody (WOW-1 Fab) which recognizes integrin $\alpha_v\beta_3$ in its high affinity state; total integrin was stained using a monoclonal $\alpha_v\beta_3$ antibody. The results are representative of two independent experiments.

Figure 4. Laulimalide did not affect VEGFR-2 tyrosine phosphorylation, but did inhibit the association of VEGFR-2 with Hsp90 and FAK, and the tyrosine phosphorylation of FAK and paxillin. Confluent HUVEC seeded on fibronectin (10 µg/ml) were starved overnight before being treated with Taxotere or laulimalide for 1 h at the indicated

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concentrations. Cells were then stimulated with VEGF (10 ng/ml) for 5 min (A), 10 min (B) or 30 min (C and D), the times having been determined in pilot studies to produce maximal phosphorylation of each respective protein; control cells (--) were not treated with VEGF or drug. Changes in the levels of phosphorylated Tyr951 (pY^{951}) of VEGFR-2 (A), Tyr397, Tyr407, Tyr576, Tyr577, and Tyr861 of FAK (B), and Tyr31 of paxillin (D) were visualized by immunoblots using phosphorylation-specific antibodies. Total FAK and paxillin levels were also determined. For immunoprecipitations (C), cell lysates from control, VEGF, and laulimalide+VEGF-treated cells were immunoprecipitated (IP) with an antibody to VEGFR-2, and then analyzed by SDS-PAGE. Changes in the levels of Hsp90 and FAK, associated with VEGFR-2, were visualized by probing with the indicated antibodies. Total VEGFR-2 levels were also determined.

Figure 5: Laulimalide inhibited basal levels (in the absence of VEGF) of tyrosine phosphorylation of paxillin. HUVEC were seeded on fibronectin (10 µg/ml), and starved overnight in M199 (0.1% FBS) before treatment with Taxotere or laulimalide for 1 hr at the indicated concentrations. Changes in paxillin phosphorylation were visualized by immunoblotting with an antibody to phosphotyrosine residue 31. Protein loading was evaluated by reprobing the membranes for total paxillin and actin.

Figure 6: Differential effects of laulimalide and Taxotere: the association of VEGFR-2 with integrin $\alpha_5\beta_1$, was blocked by Taxotere but not laulimalide, while the association of RhoA with integrin $\alpha_5\beta_1$ was blocked by laulimalide but not by Taxotere. Confluent HUVEC were seeded on fibronectin (10 µg/ml), starved overnight, and treated with the indicated concentrations of Taxotere or laulimalide for 1 h. Cells were then stimulated with VEGF (10 ng/ml) for 30 min. Cell lysates from control, VEGF, Taxotere+VEGF, and laulimalide+VEGF-treated cells were immunoprecipitated (IP) with antibodies to integrin $\alpha_5\beta_1$ or a control IgG, as indicated, and then analyzed by SDS-PAGE. Changes in the levels of VEGFR-2 (**A**) and RhoA

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(**B**) associated with integrin $\alpha_5\beta_1$ were visualized by probing with the indicated antibodies. Total integrin β_1 levels, and RhoA and actin levels (C) were also determined.

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Figure 1

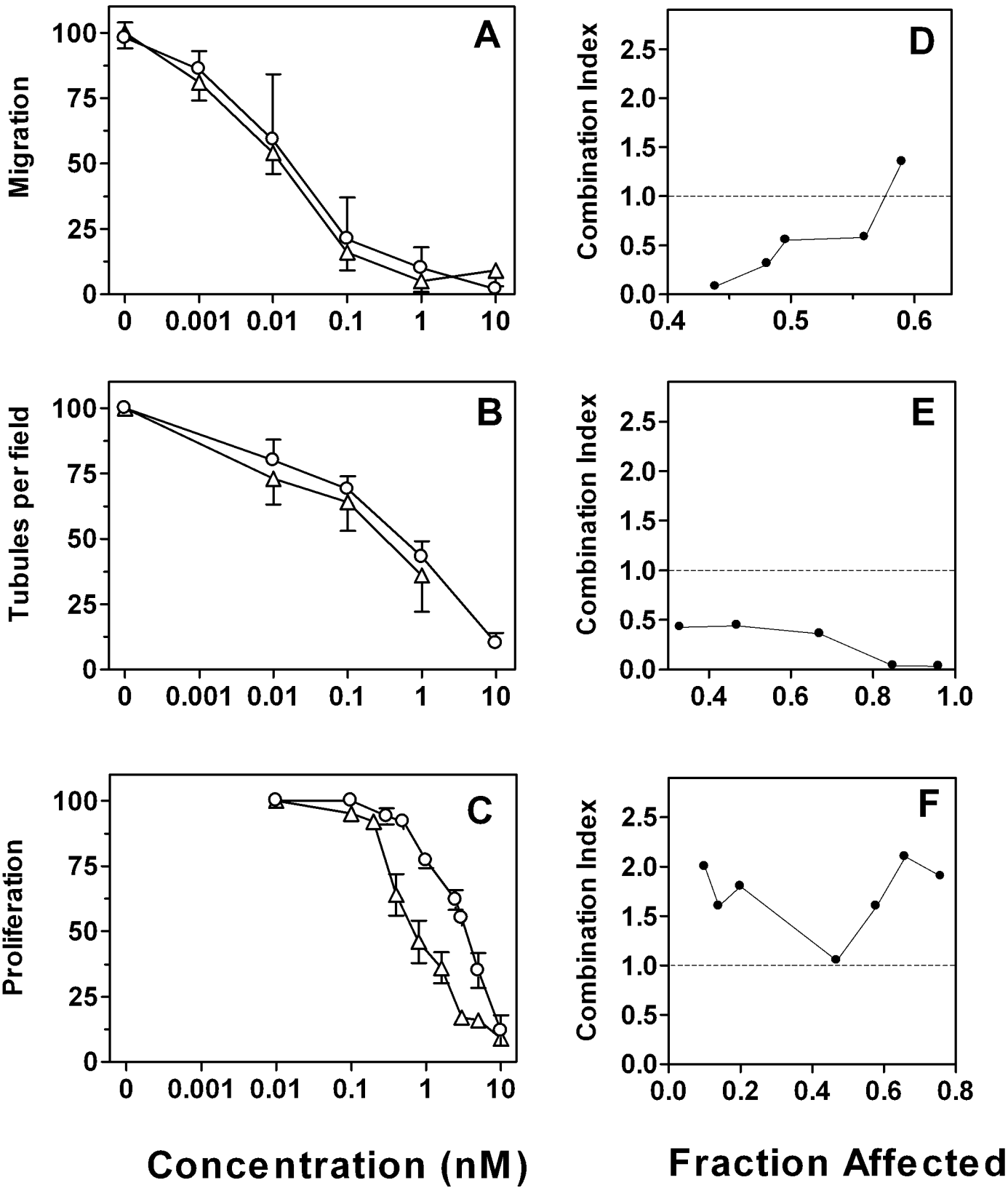


Figure 2

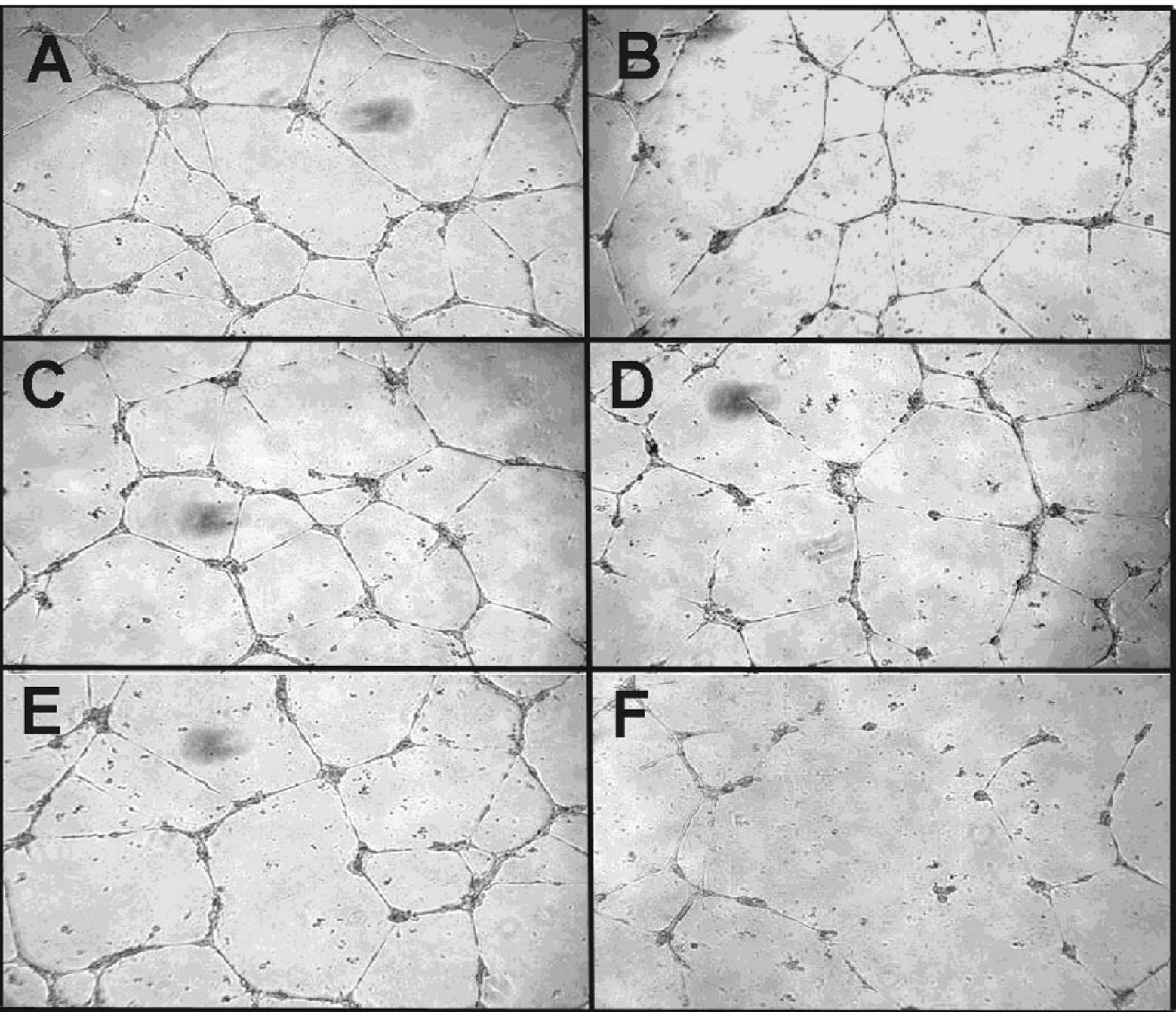


Figure 3

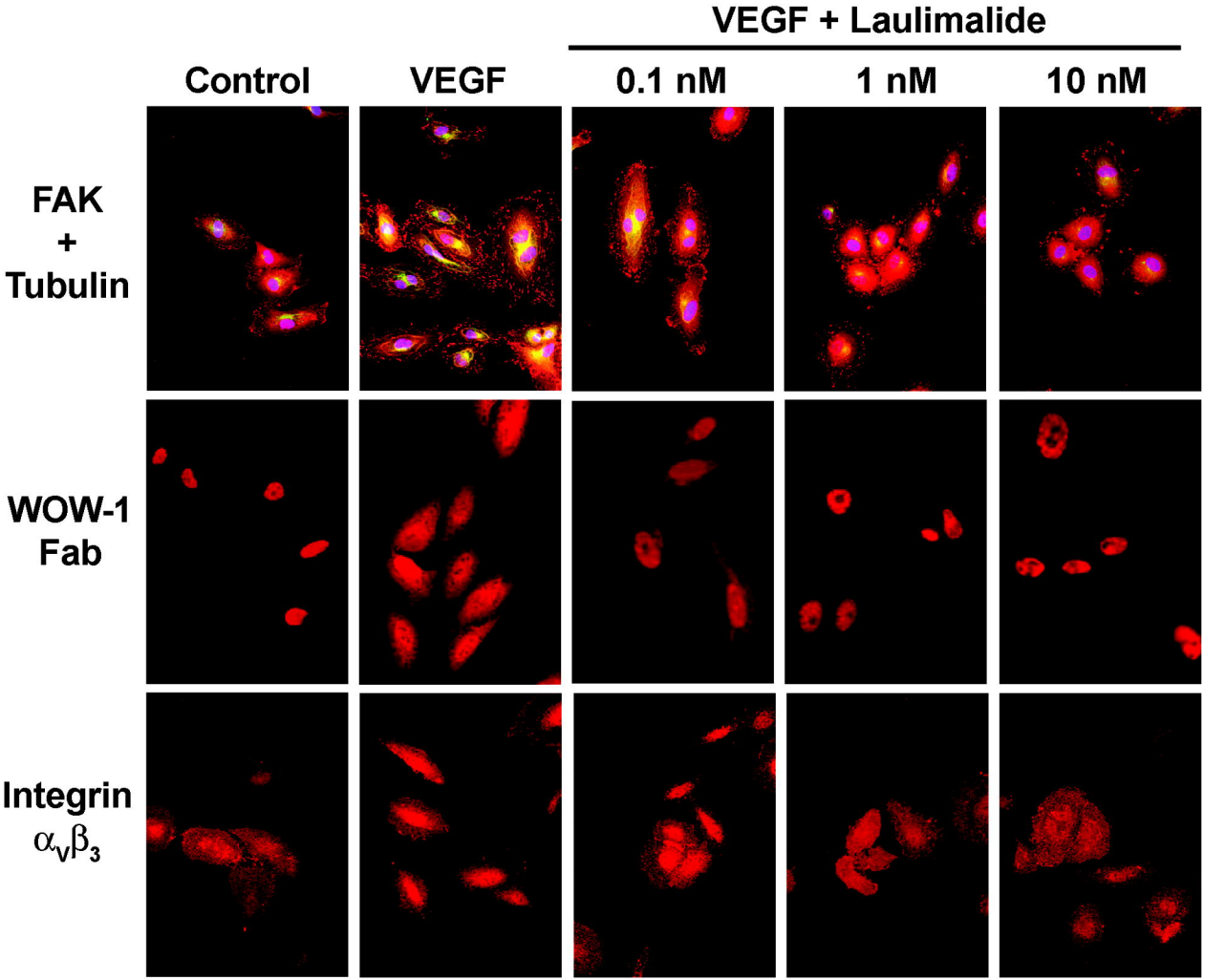

VEGF + Laulimalide
Control
VEGF
0.1 nM
1 nM
10 nM
FAK + Tubulin
WOW-1 Fab
Integrin $\alpha_V\beta_3$

Figure 4

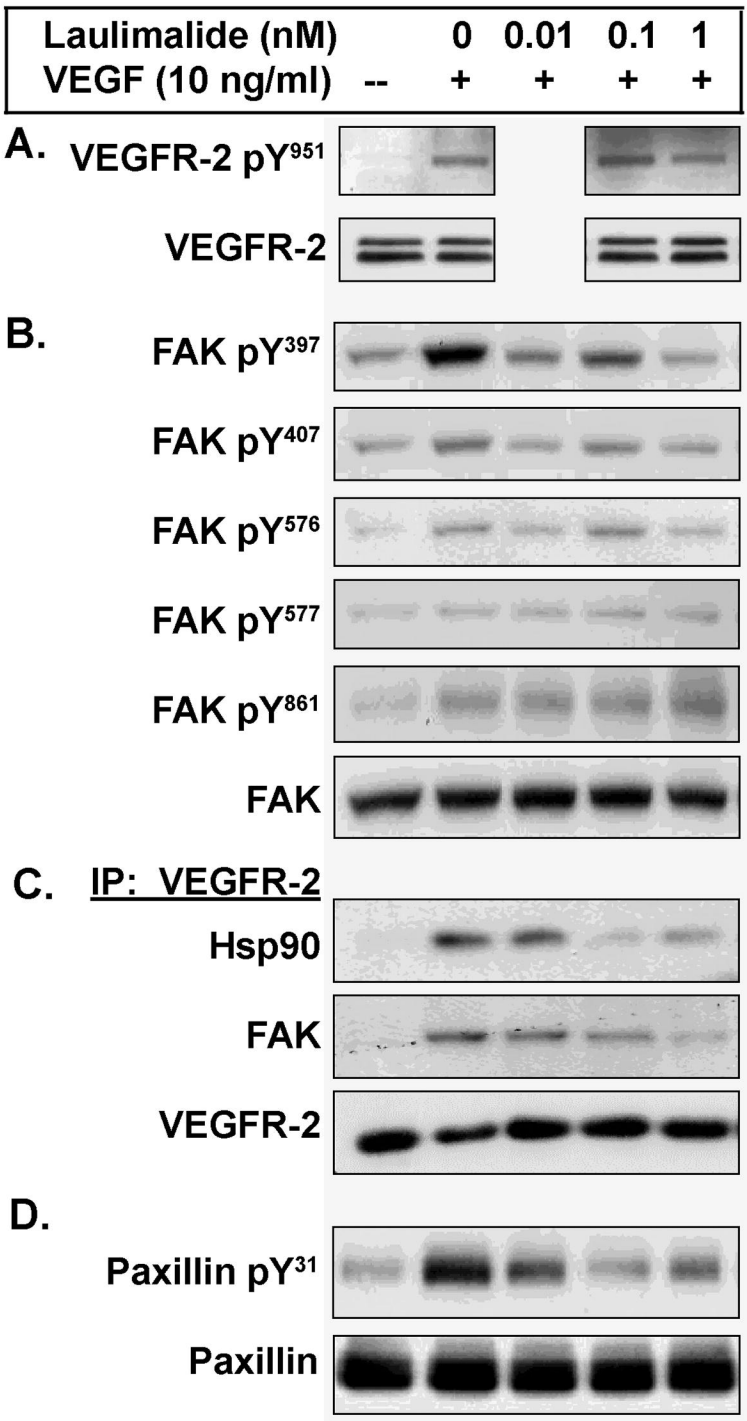

Laulimalide (nM) 0 0.01 0.1 1
VEGF (10 ng/ml) -- + + + +
A.
VEGFR-2 pY951
VEGFR-2
B.
FAK pY397
FAK pY407
FAK pY576
FAK pY577
FAK pY861
FAK
C.
IP: VEGFR-2
Hsp90
FAK
VEGFR-2
D.
Paxillin pY31
Paxillin

Figure 5

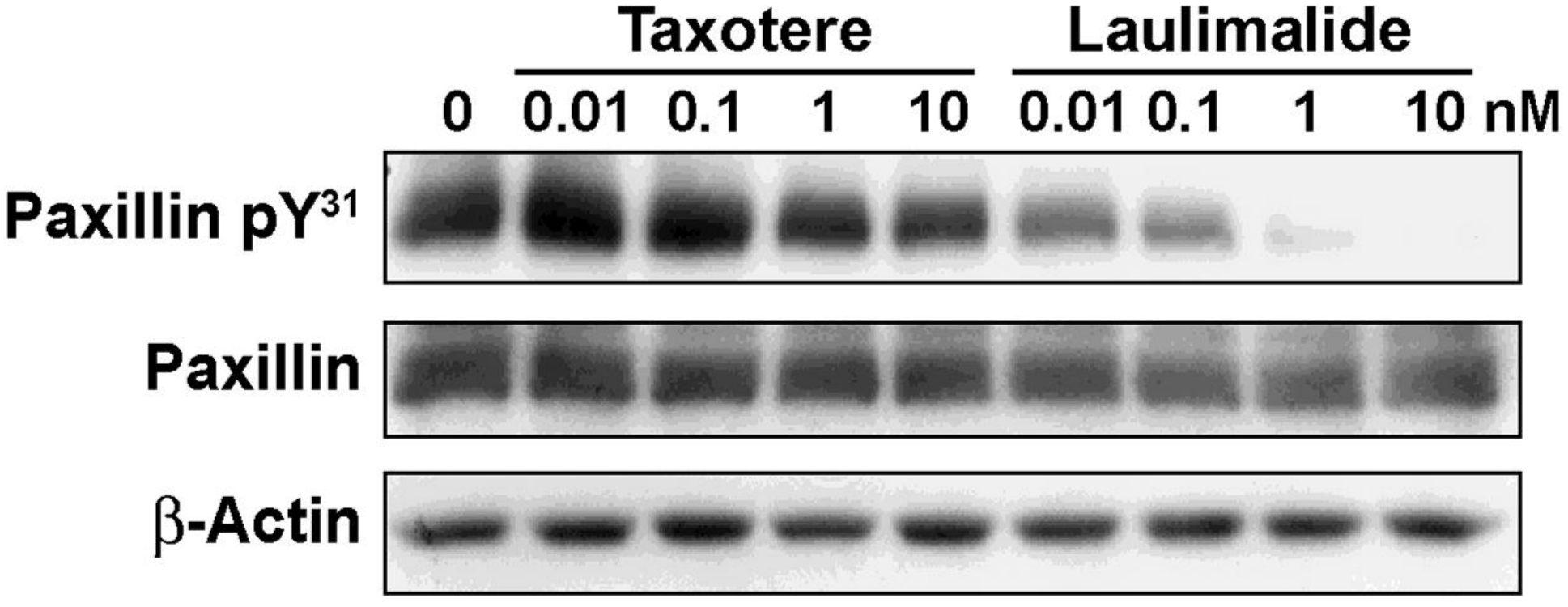
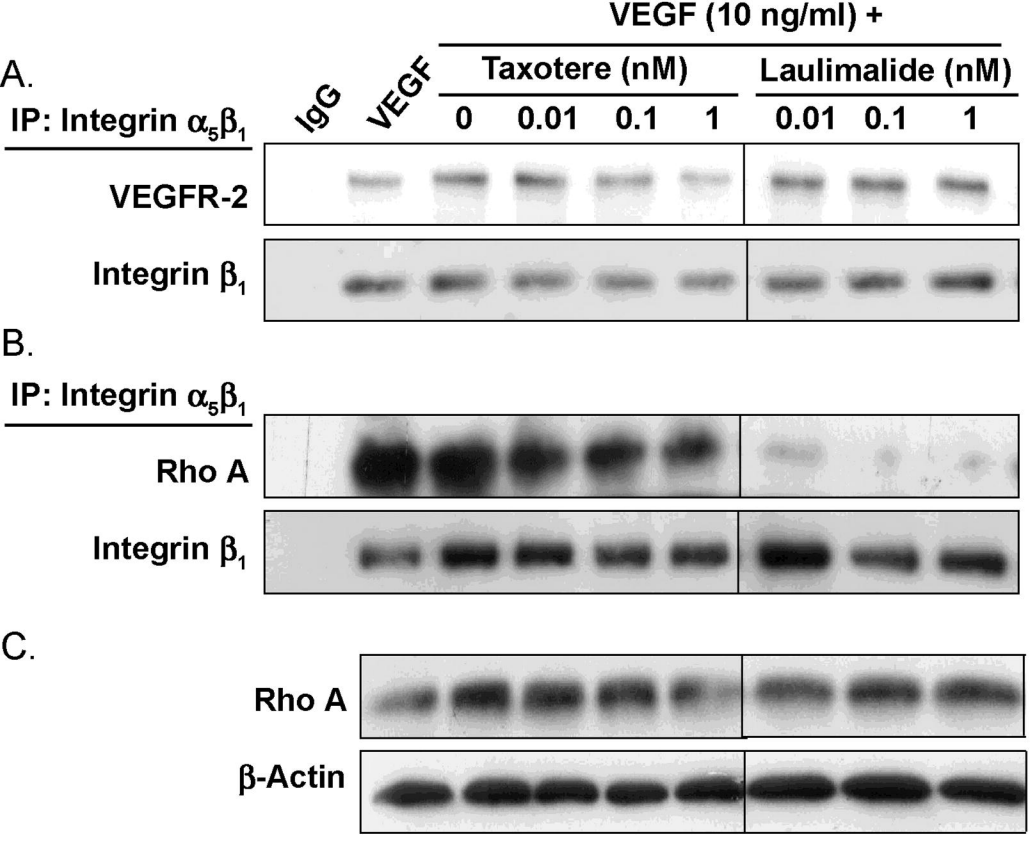

Taxotere
Laulimalide
0
0.01
0.1
1
10
0.01
0.1
1
10 nM
Paxillin pY31
Paxillin
β-Actin

Figure 6


VEGF (10 ng/ml) +
A.
IgG
VEGF
Taxotere (nM)
Laulimalide (nM)
0
0.01
0.1
1
0.01
0.1
1
IP: Integrin $\alpha_5\beta_1$
VEGFR-2
Integrin β_1
B.
IP: Integrin $\alpha_5\beta_1$
Rho A
Integrin β_1
C.
Rho A
β-Actin