Models and Technologies

An In Vivo Antilymphatic Screen in Zebrafish Identifies Novel Inhibitors of Mammalian Lymphangiogenesis and Lymphatic-Mediated Metastasis

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Abstract

The growth of new lymphatic vessels (lymphangiogenesis) in tumors is an integral step in the metastatic spread of tumor cells, first to the sentinel lymph nodes that surround the tumor and then elsewhere in the body. Currently, no selective agents designed to prevent lymphatic vessel growth have been approved for clinical use, and there is an important potential clinical niche for antilymphangiogenic agents. Using a zebrafish phenotype-based chemical screen, we have identified drug compounds, previously approved for human use, that have antilymphatic activity. These include kaempferol, a natural product found in plants; leflunomide, an inhibitor of pyrimidine biosynthesis; and cinnarizine and flunarizine, members of the type IV class of calcium channel antagonists. Antilymphatic activity was confirmed in a murine in vivo lymphangiogenesis Matrigel plug assay, in which kaempferol, leflunomide, and flunarizine prevented lymphatic growth. We show that kaempferol is a novel inhibitor of VEGFR2/3 kinase activity and is able to reduce the density of tumor-associated lymphatic vessels as well as the incidence of lymph node metastases in a metastatic breast cancer xenograft model. However, in this model, kaempferol administration was also associated with tumor deposits in the pancreas and diaphragm, and flunarizine was found to be tumorigenic. Although this screen revealed that zebrafish is a viable platform for the identification and development of mammalian antilymphatic compounds, it also highlights the need for focused secondary screens to ensure appropriate efficacy of hits in a tumor context. Mol Cancer Ther; 13(10); 1–13. ©2014 AACR.

Introduction

Cancer is now the leading cause of death worldwide. The majority of cancer-induced deaths are caused by metastatic malignancy, a process by which cancer cells spread through the body via the lymphatic or blood vasculature. The importance of lymphatic metastases is well recognized in cancer staging and treatment, with the spread of cancer cells to surrounding lymph nodes being associated with poor prognosis in many cancers (1, 2). It has been estimated that more than 80% of solid tumors metastasize, at least partially, through the lymphatic vasculature (3). Tumor cells can enter the lymphatic vasculature by either invading preexisting lymphatic vessels present in the tissue surrounding the tumor, or by promoting lymphangiogenesis and creating new lymphatic vessels within and around the tumor—a process termed tumor-induced lymphangiogenesis (4, 5). The increase in lymphatic vessel density in the tumor has been proposed to facilitate the metastatic spread of cancer cells, as it has been correlated with an increased incidence of lymph node metastases and a consequential decrease in patient survival in many cancers (3, 6). As well as being implicated in cancer metastasis, de novo lymphangiogenesis is also associated with host rejection of renal transplants (7) and corneal grafts (8).

Studies have shown that the receptor tyrosine kinases VEGFR2 and VEGFR3 as well as their ligands VEGF-A, VEGF-C, and VEGF-D play important roles in tumor-induced lymphangiogenesis (4, 9–12). It is known that inhibition of the VEGF signaling pathway reduces tumor-associated lymphangiogenesis and lymph node metastasis in animal models (13–16) and also improves the survival of corneal transplants (8). Therefore, there is a need to develop and test not only inhibitors of VEGFR/VEGF signaling but also to identify novel antilymphatic drugs that act outside of this pathway.

Zebrafish are a powerful model organism in which to study lymphangiogenesis. Lymphatic vessels can be...
readily observed in transparent embryos, and zebrafish lymphatic development shares the same molecular mechanisms as used by mammals (17–19). Zebrafish embryos, like cell-based assays, are also amenable to small-molecule drug screens (20). In zebrafish, the mechanism of lymphatic vessel development in the trunk has been well established, making it ideal for antilymphatic studies. At 36 hours postfertilization (hpf), lymphatic precursors known as secondary sprouts migrate from the posterior cardinal vein (PCV) and by 48 hpf have migrated to the horizontal myoseptum where they are termed parachordal lymphangioblasts. From here, the lymphangioblasts migrate toward and then along the arterial intersegmental blood vessels (ISV) to form the thoracic duct by 120 hpf (21). Although small-molecule drug screens using zebrafish embryos have been used to successfully identify inhibitors of mammalian blood vessel development (22–24), no antilymphatic screens have been reported to date.

One prerequisite for conducting an antilymphatic chemical screen is a specific marker for lymphatic vessels. We recently identified and characterized the expression of the zebrafish ortholog of lymphatic vessel endothelial hyaluronan receptor 1 (lyve1; ref. 25). LYVE-1 is extensively used as a marker for lymphatic vessels in mice and humans, and we found zebrafish lyve1 to be an excellent marker for lymphatic development as it is expressed in the veins and the developing lymphatic vessels (26). We have also shown that treatment of zebrafish embryos with known lymphatic inhibitors reduced or abolished the expression of lyve1 mRNA (25).

In this study, we utilized the zebrafish model to identify novel inhibitors of mammalian lymphangiogenesis. We undertook a chemical screen in which compounds were initially identified by the ability to reduce lyve1 expression in zebrafish embryos, before being passed through secondary screens to confirm that they specifically inhibited lymphatic vessel development in zebrafish. Finally, lead compounds were characterized in a mammalian model of lymphangiogenesis and their efficacy in preventing lymph node metastases was determined in a metastatic breast cancer xenograft model.

Materials and Methods

Zebrafish

The following lines were used in this study: wild-type (AB), TG(lyve1:egfp)<s>_{Tg}^{lyve1:egfp}</s> (26), TG(fli1:egfp)<s>_{Tg}^{fli1:egfp}</s> (27), and TG<(kdrl;nlsmCherry)<s>_{Tg}^{kdrl;nlsmCherry}</s> (28).

Compounds

The Prestwick Chemical Library was supplied at 2 mg/mL in DMSO. Compounds were diluted to 5 μg/mL in screening media consisting of 1 mmol/L Tris (pH 7.5) and 1x penicillin/streptomycin (Gibco) made up in E3 media. Additional compounds were sourced as follows: leflunomide (CAS #520-18-3) from Ivy Fine Chemicals, and rapamycin (CAS #53123-88-9) from Life Research and Selleck Chemicals. For animal studies, the following vehicles were used: 2.5% ethanol (Merck), 5% polyethylene glycol 400 (Sigma), and 5% Tween-80 (Sigma) for rapamycin; 20% 2-hydroxypropyl-β-cyclodextrin (Sigma) for flunarizine; and 1% and 1.5% carboxymethylcellulose (Sigma) for kaempferol and leflunomide, respectively.

Whole-mount in situ hybridization

In situ hybridization was performed using a lyve1 antisense probe (25) in the Biolane HTI in situ robot (Hölle and Hüttner), as described previously (29).

Zebrafish image analysis and statistical analysis

Embryos were imaged as described (30). Thoracic duct formation at 120 hpf and secondary sprout formation at 36 hpf were scored as described previously (26). Lymphangioblast cell tracking for the first 8 hours following their emergence from the PCV was performed manually using ImageJ (NIH, Bethesda, MD). Statistical analysis was performed using Prism 5.0 software (GraphPad Software Inc). Significance was determined by Mann–Whitney tests.

Mouse studies

All mouse experiments followed protocols approved by the Animal Ethics Committee of the University of Auckland (Auckland, New Zealand). Age-matched female C57BL/6 mice and NIH-III nude mice (NIH-Lys<sup>kb</sup>-Foxn1<sup>nu</sup>Btk<sup>–/–</sup>) weighing between 18 g and 25 g were provided by the Vernon Jansen Unit, University of Auckland.

Matrigel plug assay

Female C57BL/6 mice were anesthetized with ketamine (100 mg/kg; Parnell Living Science) and xylazine (10 mg/kg; Phoenix Pharmaceuticals Inc), followed by shaving of the left flank and subcutaneous injection of 500 μL Matrigel (Becton Dickinson) supplemented with VEGF-C (1 μg/mL; R&D Systems). Mice were randomly assigned to treatment groups dosed at maximum tolerated dose (MTD; 200 mg/kg kaempferol, 200 mg/kg leflunomide, and 30 mg/kg flunarizine; n = 4 mice/group) 1 hour post Matrigel implantation, and then daily for a further 9 days. Plugs were then harvested and frozen in liquid nitrogen for cryostat sectioning into 5-μm fresh-frozen sections.

Cell culture

MDA-MB-231-luc D3H2LN cells were supplied by Caliper Life Sciences (November 2009) and authenticated by short tandem repeat profiling at CellBank Australia in September 2011. Cells were confirmed to be Mycoplasma-negative by PCR testing (Boehringer Mannheim) and were passaged in culture for less than 6 months after authentication. Cells were cultured in α-minimum essential media containing 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, and incubated at 37°C in 5% CO₂.
37°C in a 5% CO₂ humidified incubator. Cells were used in experiments during the exponential phase of growth (60%–80% confluency).

Orthotopic xenograft study
The fourth mammary fat pads of NIH III nude mice were inoculated with 3 x 10⁶ MDA-MB-231-luc D3H2LN cells suspended in Matrigel and PBS. At 48 hours, mice with detectable tumor cells were recruited into the intervention regimen, in which daily dosing began (control; n = 4 mice/group; 150 mg/kg kaempferol, 40 mg/kg flunarizine, and 5 mg/kg rapamycin; n = 6 mice/group). The remaining mice were left untreated until week 4 when mice with established tumors were recruited into the intervention regimen and daily dosing began (control; n = 4 mice/group; 150 mg/kg kaempferol and 40 mg/kg flunarizine; n = 6 mice/group). Control prevention and intervention regimens were pooled for analysis. Primary tumor growth was measured every 2 to 3 days with digital calipers. At 7 to 8 weeks posttumor inoculation, mice were euthanized and their primary tumors, selected lymph nodes (brachial, axillary, and inguinal), pancreases, diaphragms, and any other tissues with visible metastases were harvested. Tissues were then fixed in 10% neutral-buffered formalin, paraffin-embedded, and 5 µm fixed tissue sections prepared for IHC.

Bioluminescence imaging
Mice were anesthetized with isoflurane (Lunan Pharmaceutical Company) and then imaged with the IVIS Kinetic imager (Caliper Life Sciences) approximately 10 minutes after subcutaneous administration of 150 mg/kg D-Luciferin firefly potassium salt (Gold Biotechnology) in PBS. Imaging was carried out 24 hours following tumor cell inoculation to confirm the success of the procedure, and then weekly from week 3. After week 4, mice were probed for secondary metastatic signals by shielding the primary tumor with black paper and taping back the forelimbs to expose the brachial/axillary lymph node areas. Tissues harvested postmortem were imaged ex vivo in 24-well tissue culture plates containing 300 µg/mL luciferin.

Immunohistochemistry
For both tumor and Matrigel sections, lymphatic vessels were visualized by IHC using primary rabbit anti-mouse LYVE1 (1:150; Angiobio) and rat anti-mouse F4/80 (1:50; Invitrogen) antibodies, and secondary goat anti-rat AlexaFluor 488 and goat anti-rabbit AlexaFluor 568 antibodies (1:200; Invitrogen), followed by counterstaining with DAPI (Invitrogen). Imaging of sections was carried out using a Zeiss Axio Imager Z2 microscope for whole section scanning and an Olympus FV1000 confocal microscope for high-resolution imaging. Lymphatic vessel density was calculated as the number of LYVE1/+/F480+ vessels per µm² of either tumor or Matrigel plug and graphed as a percentage of control. A minimum of two sections covering the entire area of the tumor or Matrigel plug was used per animal. Statistical analysis was performed using Prism 6.0 software (GraphPad Software Inc), and significance was determined by one-way ANOVA.

Results
A chemical screen in zebrafish identified drugs with antilymphatic activity
The Prestwick Chemical Library, containing 1,120 compounds (the majority of which are FDA-approved drugs) was selected for our screen. Using our antilymphatic screening assay (see Supplementary Methods), we identified 12 compounds, a hit rate of 1.1%, which reduced or abolished lyve1 expression without adverse side-effects such as developmental delay or tissue necrosis. The mTOR inhibitor, rapamycin, was included as a positive control as it is a known inhibitor of both mammalian and zebrafish lymphangiogenesis (25, 31, 32). A secondary screen for thoracic duct formation using lyve1:egfp embryos (26) showed that six compounds displayed antilymphatic activity in zebrafish: the flavonoid kaempferol, the dihydroorotate dehydrogenase inhibitor leflunomide, 2 diphenylpiperazine calcium channel antagonists, flunarizine and cinnarizine and 2 statins, lovastatin, and simvastatin (Fig. 1A and Supplementary Fig. S1). The identification of statins as antilymphatic was expected as both compounds were recently shown to have antilymphatic properties (33). The secondary screen using fli1:egfp embryos identified kaempferol, leflunomide, cinnarizine, and flunarizine as agents that prevented lymphangiogenesis in the zebrafish without disrupting blood vessel development, whereas lovastatin and simvastatin disrupted ISV formation at the screening dose of 5 µg/mL. When the concentration of each statin was reduced so that they no longer blocked ISV formation, the thoracic duct formed normally (Supplementary Fig. S2). As lovastatin and simvastatin are not specific inhibitors of lymphangiogenesis, we did not consider these compounds for further analysis.

Kaempferol, leflunomide, cinnarizine, and flunarizine reduced lyve1 expression in our in situ screen and prevented thoracic duct formation in either fli1:egfp or lyve1:egfp embryos (Fig. 1B and C and Supplementary Fig. S1). Using thoracic duct formation as a readout of drug efficacy, we established a response curve and MTD for each compound in zebrafish embryos (Fig. 1C). The MTD for kaempferol was 30 µmol/L, leflunomide 4 µmol/L, cinnarizine 28 µmol/L, and flunarizine 5 µmol/L. These doses were used for all subsequent zebrafish experiments. We also tested the active metabolite of leflunomide, A77 1726 (34, 35), and found that it also inhibited lymphatic vessel development with an MTD of 2 µmol/L.

Kaempferol and leflunomide inhibit the sprouting of zebrafish lymphatic vessels
To establish which aspect of lymphatic vessel development each drug was inhibiting, we imaged the trunk region of lyve1:egfp embryos at 36 hpf and 48 hpf. We scored the number of secondary sprouts at 36 hpf and...
found that embryos treated with either kaempferol, leflunomide, or A77 1726 had reduced numbers of secondary sprouts, whereas cinnarizine or flunarizine-treated embryos were normal (Fig. 2A and B). We also scored the number of parachordal lymphangioblasts at 48 hpf and found that kaempferol, leflunomide, and A77 1726 caused a reduction in lymphangioblasts, consistent with a defect in secondary sprouting. However, both cinnarizine and flunarizine-treated embryos had normal lymphangioblast formation (Fig. 2C and D).

Confirmation of these phenotypes was obtained by time-lapse imaging of lyve1:egfp embryos, in the absence
Supplementary Video S1) and presence of the antilymphatic compounds, from 30 to 49 hpf. In this way, we confirmed that kaempferol causes a defect in secondary sprouting, as no secondary sprouts were observed in three time-lapse experiments (Fig. 3; Supplementary Video S2). Leflunomide caused secondary sprouts to migrate at half the speed observed in control embryos (4.9 \mu m/hour in leflunomide compared with 11.4 \mu m/hour in control) and each sprout had an abnormal morphology, with multiple vascular tips (Fig. 3; Supplementary Video S3). Secondary sprouts in embryos treated with flunarizine looked normal and migrated at comparable speed to controls (Fig. 3; Supplementary Video S4). Our results suggest that kaempferol and leflunomide inhibit the process of lymphatic sprouting from the veins and the type IV calcium channel agonists, cinnarizine and flunarizine, inhibit later stages of lymphatic vessel formation.

**Kaempferol inhibits VEGFR kinase activity**

The IC_{50} values for kaempferol, leflunomide, A77 1726, and flunarizine were determined against human VEGFR1, VEGFR2, and VEGFR3. Only kaempferol caused appreciable inhibition of VEGFR kinase activity with an IC_{50} of 8.37 \mumol/L against VEGFR2 and 19 \mumol/L against VEGFR3 (Supplementary Table S1). These data suggest that the antilymphatic activity we observe...
following kaempferol treatment is most likely through the inhibition of VEGFR2/3 signaling and that leflunomide and flunarizine function through other pathways.

**Flunarizine causes lymphangioblast cell death in zebrafish**

To determine the mechanism by which the type IV calcium channel inhibitors prevent lymphatic vessel formation, we imaged lyve1:egfp;kdrl:nls:mcherry embryos exposed to flunarizine at later stages of lymphatic development, from 48 hpf until 68 hpf. We took advantage of residual kdrl expression in parachordal lymphangioblasts and used a nuclear-localized kdrl transgenic, kdrl:nls: mcherry, to fate-map lymphangioblasts exposed to flunarizine. By tracking the nuclei of lymphangioblasts (lyve1 and kdrl positive) throughout three independent time-lapse experiments, we observed that in embryos exposed to flunarizine, on average 37% of their lymphangioblasts underwent nuclear fragmentation and then subsequently lost lyve1 and kdrl expression, indicative of cell death by apoptosis. In contrast, we never observed any lymphangioblast cell death in control embryos (Fig. 4; Supplementary Videos S5 and S6). Next, we performed terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) to identify cells undergoing apoptosis at 60 hpf in both control and flunarizine-treated embryos. We found an average of 2.0 TUNEL-positive lymphangioblasts per embryo with flunarizine compared with 0.1 in control embryos (Supplementary Fig. S3). Our data suggest that flunarizine, while not affecting initial specification and migration of lymphangioblasts, causes them to undergo apoptosis, leading to defects in lymphatic vessel development.

**Antilymphatic drugs identified in zebrafish inhibit mammalian lymphangiogenesis**

To confirm that the antilymphatic compounds identified could also inhibit mammalian lymphangiogenesis, they were administered to C57BL/6 mice injected with Matrigel plugs supplemented with recombinant VEGF-C.
The amount of lymphatic vessel growth into the plug was determined by immunostaining with anti-LYVE1. To rule out any confounding LYVE1 staining in macrophages (36), we also performed immunostaining with an F4/80 antibody against macrophage cells and selected LYVE1-positive F4/80-negative cells as being lymphatic. As expected, rapamycin was able to significantly reduce the number of lymphangioblasts that die per time-lapse experiment (from 48 hpf to 68 hpf) in embryos treated with 1% DMSO control. Scale bar, 20 μm.

**Kaempferol reduces lymph node metastases but increases systemic metastases in orthotopic breast cancer xenografts**

To test whether the antilymphatic activity of kaempferol and flunarizine could reduce lymphatic metastasis, we used an orthotopic breast cancer xenograft model in which metastatic human breast cancer cells expressing luciferase (MDA-MB-231-luc-D3H2LN) were injected into the mammary fat pad of NIH-III nude mice. Animals were treated with drugs by two different regimens: prevention treatment, in which mice were dosed daily with antilymphatic drugs 48 hours posttumor implantation and intervention treatment, which was initiated 28 days posttumor implantation, just before the onset of metastases detection in controls. The dose of flunarizine was increased to 40 mg/kg to attempt to enhance efficacy, whereas kaempferol was reduced to 150 mg/kg to improve tolerance. Both drugs, and the control drug rapamycin, were well tolerated and did not cause any adverse body weight change in the NIH-III nude mice (Supplementary Fig. S4). Rapamycin inhibited primary tumor growth, whereas kaempferol had minimal effect on tumor volume and flunarizine promoted tumor growth in both prevention and intervention groups as determined by caliper measurement and bioluminescence imaging. (Fig. 6A and B and Supplementary Fig. S5). Primary tumors were removed at the conclusion of treatment, 7 to 8 weeks after tumor cell inoculation, to assess the density of tumor-associated lymphatics (which includes both peritumoral and intratumoral lymphatic vessels) by LYVE1-positive F4/80-negative staining. Kaempferol was effective at reducing the density of tumor-associated lymphatics in both the prevention and intervention groups and any lymphatics we observed were often smaller and appeared collapsed compared with controls. In contrast, tumors from flunarizine-treated mice had similar levels of tumor-associated lymphatics to control tumors (Fig. 6C and D).

In addition to the primary tumor, the brachial, axillary, and inguinal lymph nodes surrounding the tumors as well as the pancreas and diaphragm were removed and were imaged ex vivo for any luciferase activity that would indicate that cancer cells had metastasized (Fig. 7A). The frequency of lymph nodes with a positive bioluminescent signal was significantly reduced in rapamycin and kaempferol prevented groups relative to controls; however, flunarizine-treated mice had a significant increase in the number of lymph nodes with a bioluminescent signal. In the intervention arm, kaempferol was similarly effective, reducing the frequency of lymph nodes with a positive bioluminescent signal (Fig. 7B).

Although kaempferol was effective at reducing lymph node metastases, 80% of mice treated with kaempferol had MDA-MB-231-luc-D3H2LN cells present in the pancreas and diaphragm in both the prevention and intervention regimens, compared with 10% of control mice as determined by bioluminescence imaging. No evidence of bioluminescence was observed in pancreas or diaphragm tissue in rapamycin-treated mice in the prevention regimen (Fig. 7C) or in flunarizine intervention mice, but was in 50% of flunarizine prevention mice. The presence of cancer cell infiltration into lymph node, pancreas, and diaphragm tissue was confirmed by histopathological evaluation of hematoxylin and eosin (H&E)-stained sections (Fig. 7D).
reduced with kaempferol treatment, we observed an increase in the incidence of metastases in the pancreas and diaphragm. It is unclear whether the increase in systemic metastases results from a compensatory mechanism to overcome the inhibition of tumor lymphatic growth, or if kaempferol is somehow promoting nonlymphatic spread of cancer cells. It is also uncertain whether these metastases are the result of hematogenic spread or the local invasion of tumor cells. Regardless, these results suggest that antilymphatics may be best given in conjunction with other compounds that prevent tumor growth. Previous work has shown that kaempferol consumption has been associated with reduced risk of developing many cancers (38–40) and has also been shown to reduce VEGF expression in human cancer cell lines (41, 42). To our knowledge, this study is the first to report VEGFR inhibition and antilymphatic activity for kaempferol.

Our second antilymphatic compound, leflunomide, is an inhibitor of the enzyme dihydroorotate dehydrogenase (DHODH), involved in pyrimidine biosynthesis (43, 44). Leflunomide is used as a disease-modifying drug to treat patients with rheumatoid arthritis (45) and has also been shown to have antitumor properties in models of melanoma (46). In this study, we found that leflunomide and its active metabolite, A77 1726, are able to inhibit secondary sprouting of lymphatic precursors in zebrafish embryos and it is also able to inhibit lymphangiogenesis in a murine Matrigel plug assay. We believe that leflunomide is likely to be acting as an antilymphatic drug through its ability to inhibit DHODH as the low micromolar doses we used in zebrafish are unable to inhibit human VEGFR signaling and are consistent with leflunomide functioning as an inhibitor of pyrimidine biosynthesis (35, 46). It is possible that as lymphangioblasts migrate and remodel to form vessels, they are also sensitive to reduced levels of pyrimidines and therefore by inhibiting de novo pyrimidine biosynthesis in the lymphangioblasts, leflunomide is able to prevent lymphatic vessel formation.

The final antilymphatic hits, cinnarizine and flunarizine, are both diphenylpiperazine calcium channel antagonists. Flunarizine is a fluorine analogue of cinnarizine. Both drugs are classified as type IV, or non-selective, calcium channel antagonists (47). The fact that both flunarizine and cinnarizine were identified in our screen strongly suggests that this class of calcium channel antagonist has efficacy as an antilymphatic compound. A previous screen identified members of the class I calcium channel blockers, felodipine and nicardipine, as antilymphatic in Xenopus embryos, although these drugs did not inhibit lymphangiogenesis in in vitro assays (48). Neither felodipine nor nicardipine was present in our chemical library, but we did screen other class I calcium inhibitors (nimodipine and nifedipine). However, they did not display antilymphatic activity at our screening dose, suggesting that class IV calcium channel antagonists are more effective antilymphatic compounds than the class I inhibitors.

Flunarizine did not inhibit secondary sprouting in the zebrafish, but rather it caused the parachordal lymphangioblasts to undergo apoptosis. Like leflunomide, flunarizine does not inhibit human VEGFR activity at
micromolar concentrations and therefore could be a useful agent to complement existing VEGFR3 inhibitors that, like kaempferol, target the early steps of lymphatic sprouting from veins. Although flunarizine was also able to inhibit mammalian lymphangiogenesis in a Matrigel plug assay, its efficacy as a potential antimetastatic agent was difficult to determine as it promoted tumor growth. Flunarizine has been shown to increase blood flow into tumors (49, 50). It is possible that increased tumor blood flow induced by flunarizine could account for the tumorigenic properties we observed. Our data suggest that flunarizine is not a suitable antilymphatic agent for the...

Figure 6. The effect of drugs on tumor growth and tumor lymphangiogenesis. A and B, tumor volume in NIH-III nude mice, orthotopically xenografted with MDA-MB-231-luc-D3H2LN cells, in which daily drug dosing began (A) 48 hours posttumor implantation (prevention group) or (B) 28 days posttumor implantation (intervention group). Error bars, ± SEM. C, images of tumor sections costained with anti-LYVE-1 (red) and anti-F4/80 (green). Lymphatic vessels are LYVE-1-positive, F4/80-negative (arrows). The tumor (T) boundary is marked with a dotted line. D, quantitation of intratumoral and peritumoral lymphatic vessel density in tumors. Error bars ± SD. *, P < 0.05; **, P < 0.01 by one-way ANOVA. Scale bar, 100 μm.
prevention of metastatic disease but it could still have utility in the treatment of other lymphatic-based pathologies such as the host rejection of corneal grafts.

This study has shown that zebrafish is a viable platform for the identification and development of mammalian antilymphatic compounds; however, we observed effects of the two drugs tested in our xenograft model that suggest they would not be useful in a tumor setting. This is not surprising given that we isolated antilymphatic compounds solely on the ability to prevent lymphangiogenesis, while the influence of these drugs on tumor growth and cancer cell migration was not assessed. Any
future zebrafish antilymphatic screens should utilize additional secondary screens to ensure better translation of antilymphatic drugs in the prevention of metastasis. Possible secondary screens could ensure that antilymphatic compounds do not promote tumor growth or migration by conducting in vitro cancer cell line growth and migration assays.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Grant Support
This work was supported by grant number UOAX0813 from the New Zealand Ministry of Business, Innovation and Employment (to P.S. Crosier).

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Received June 3, 2014; revised July 9, 2014; accepted July 13, 2014; published OnlineFirst July 22, 2014.

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Molecular Cancer Therapeutics

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Mol Cancer Ther  Published OnlineFirst July 22, 2014.

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doi:10.1158/1535-7163.MCT-14-0469-T

Supplementary Material  Access the most recent supplemental material at:
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