models using human tumor xenografts of a variety of histological origins. Efforts to identify inhibitors of these receptor tyrosine kinases led to the identification of BAY 57-9352. This compound is a potent, orally active inhibitor of VEGFR-2 and PDGFR tyrosine kinase activity, cellular receptor autophosphorylation and VEGF- and PDGF-stimulated mitogenesis *in vitro*. The present studies describe the investigation of major metabolic pathways of BAY 57-9352 following incubations with liver preparations, the elucidation of metabolizing human cytochrome P450 (CYP) isoforms involved and the inhibitory and inductive potential of BAY 57-9352 on major human CYP isoforms. The main phase I metabolic pathways in man were assessed *in vitro* using human liver microsomal preparations. Additionally, phase II biotransformations were assessed in a sandwich model with cultured hepatocytes. CYP 3A4, 2C8, 2C19 and 1A2 and glucuronosyl transferase were found to be the major metabolizing enzymes. These data indicate that BAY 57-9352 metabolism is mediated by multiple enzymes.
The potential of BAY 57-9352 to induce the human P450 isoforms CYP 1A2 and 3A4 was investigated in cultured human hepatocytes of five different donors. No inductive effects on CYP 1A2 and 3A4 were observed after treatment of human hepatocytes with concentrations up to 7 μM BAY 57-9352 in the hepatocyte medium. These results provide evidence that BAY 57-9352 is not an inducer of CYP1A2 and 3A4 up to a concentration, which is markedly higher than therapeutic plasma concentrations of BAY 57-9352 in preclinical models. In addition, no major CYP enzyme inhibition was observed. Taken together, the data indicate that clinical drug-drug interactions through inhibition or induction of CYP enzymes by concomitantly administered drugs are unlikely. Based on a favorable pharmacological and *in vitro* metabolic profile, BAY 57-9352 has advanced to Phase 1 clinical trials as an anti-angiogenic agent.

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Tyrosine kinase inhibition as a strategy to suppress growth of primitive endothelial cells

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Blockade of receptor tyrosine kinases (RTKs) is an increasingly important area of cancer research because RTKs are ubiquitous, and mutated forms often give rise to cells that are constitutively activated. Imatinib, an inhibitor of the split RTK family including c-kit and vascular endothelial growth factor receptor (VEGFR), has had a major impact on the treatment of several cancers associated with dysregulated activation of RTKs. Angiogenesis, the formation of new blood vessels from established vasculature, results from proangiogenic stimuli, such as vascular endothelial growth factor (VEGF), stem cell factor (SCF), and others, acting on mature endothelial cells. To begin to test the hypothesis that blocking RTKs on endothelial cells could suppress angiogenesis in cancer, we developed a unique endothelial xenograft model utilizing neoplastic canine endothelial cells (SB-HSA) derived from a malignant angiosarcoma in a dog (Akhtar et al, Neoplasia 6:106, 2004). All studies were done with prior approval of the UW Animal Care Committee. Canine angiosarcomas are comprised of primitive endothelial cells and recapitulate many features characteristic of mitotically active endothelial cells (Fosmire et al, Lab Invest 84:562, 2004). The cells used in our xenograft model express VEGFR-2 and c-kit, as well as their ligands, VEGF and SCF. NOD-SCID mice injected with SB-HSA cells and treated with imatinib developed statistically smaller tumors compared to untreated control mice. Therefore, blocking RTKs in this model had an important therapeutic benefit in arresting the growth of mitotically active endothelial cells. To extend these findings and to begin to learn more about the pathways affected, we have examined spontaneous angiosarcomas from multiple dogs and found them to express c-kit uniformly. We are pursuing investigations intended to clarify expression and activation of other RTKs including VEGFR-2 and platelet-derived growth factor as possible targets of imatinib in this model. Taken together, we have shown a promising effect of RTK inhibition in a novel angiogenesis model that may have potentially broader applications in cancer. Furthermore, the natural occurrence of canine angiosarcoma may provide a unique opportunity to evaluate RTK inhibition as a strategy to suppress the angiogenesis of malignancy in an out bred animal model. (Supported by NIH CA86264, Morris Animal Foundation D03CA-71, Canine Health Foundation 2025, and a UW Companion Animal Fund grant)

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Toxin-VEGF fusion protein inhibits tumor growth

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Growth of primary tumor and metastatic lesions beyond few millimeters requires neovascularization that combines angiogenesis and vasculogenesis. The critical question in attacking neovascularization is how to target the tumor-associated vasculature while sparing the normal quiescent vasculature. Endothelial cells in tumor vasculature overexpress VEGFR-2, a major receptor for vascular endothelial growth factor (VEGF), which is expressed only at low levels in quiescent vasculature. To target only those endothelial cells that overexpress VEGFR-2, we have constructed a novel toxin-VEGF fusion, named SLT-VEGF by fusing the catalytic subunit A of Shiga-like toxin I (SLT) to the 121 amino acid-long splice variant of VEGF. To obtain an "FDA-friendly" protein, the SLT-VEGF coding sequence was cloned into the pET29a(+) bacterial expression vector (Novagen) carrying a kanamycin resistance gene. The resulting protein contains 293 aa of the full-length SLT subunit A fused to 121 aa of the human $VEGF_{121}$ via a 7-aa linker GTDDDDK. SLT-VEGF is purified from inclusion bodies to >95% purity with a yield of >5 mg/L. SLT-VEGF selectively kills (IC_{50} ~0.07 nM) growing endothelial cells overexpressing VEGFR-2, but not quiescent endothelial cells, or cells expressing low levels of VEGFR-2. SLT-VEGF retains full activity after storage for more than a year at −70ºC, one month at +4ºC, or seven days at room temperature.
SLT-VEGF activity *in vivo* was tested using PC3 human prostate tumors in Ncr nu/nu mice and 4T1 mouse mammary carcinoma tumors in Balb/c mice. Mice (n = 15) received five bi-weekly injections of SLT-VEGF, 0.05 mg/kg/injection (1 μg/mouse/injection). This SLT-VEGF treatment significantly inhibited tumor growth in both models while inducing only transient discomfort in mice and a low-titer anti-SLT antibody response. Mice bearing 4T1 tumors were also treated with different SLT-VEGF treatment regimens and tumor growth for every individual mouse was analyzed. By plotting tumor growth rates for individual mice as linear regression curves, responder and non-responder mice could be clearly distinguished in each group. We found that the proportion of mice whose tumors respond to SLT-VEGF treatment increases with an increasing cumulative dose of SLT-VEGF.
To establish the mechanism of SLT-VEGF action, the protein was injected into 4T1 and PC3 tumors that were excised 24 h later, sectioned and stained for apoptotic cells with a TUNEL kit and for markers of endothelial cells, such as VEGFR-2 and PECAM. Judging by co-localization of apoptotic and endothelial markers, SLT-VEGF induces apoptosis preferentially in endothelial cells overexpressing VEGFR-2.
We expect that the combination of highly potent and selective cytotoxicity *in vivo*, convenience of FDA-friendly production, and excellent stability would make SLT-VEGF a valuable candidate for clinical development.

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Augmentation of radiation response in upper aero-digestive tract tumors with the vascular targeting agent ZD6126

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Background: Epithelial tumors of the upper aero-digestive tract result in particularly high morbidity and mortality. Angiogenesis plays an essential role in epithelial tumor growth and metastasis. We have performed preclinical studies to investigate the anti-tumor effects of radiation combined with ZD6126, a novel agent targeting the microtubules of endothelial cells, in head and neck (H&N) and lung cancer cell lines. The hypothesis is that tumor control may be enhanced by simultaneously targeting tumor (radiation) and tumor vasculature (ZD6126).
Methods: To characterize specific cellular effects and anti-tumor activity of the investigational regimen, a series of *in vitro* studies using endothelial cells (HUVEC), and *in vivo* studies in athymic mice bearing human lung (H226, A549) and H&N (SCC1) tumor xenografts were performed.
Results: Exposure to ZD6126 results in clear morphologic changes in HUVEC and inhibits HUVEC growth in a dose dependent manner. ZD6126 also inhibits the process of capillary-like network formation in HUVEC. Flow cytometry analysis using propidium iodide staining indicates that ZD6126, with or without radiation, results in cell cycle arrest in G2/M. ZD6126 augments radiation-induced apoptosis in HUVEC as measured by caspase assay. *In vivo* matrigel angiogenesis assay demonstrates that the anti-vascular effect of ZD6126 is enhanced by combination with radiation. In tumor xenografts, extensive necrosis is observed in H226 tumors as early as 24 hours following the injection of ZD6126. Experiments in athymic mice bearing SCC1 tumor xenografts demonstrate that the combination of