11 were given in combination. Moreover, the therapeutic effect of S-3304 in combination with carboplatin or paclitaxel was demonstrated in the solid tumor model of B16-BL6 murine melanoma cells. In conclusion, S-3304 has a potential for clinical use. All animal studies were approved by the Animal Care and Use Committee prior to initiation of the studies.

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A phase I study of the novel high affinity VEGF blocker VEGF trap in patients with refractory solid tumors and lymphoma

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VEGF Trap is a fusion protein consisting of portions of the human Vascular Endothelial Growth Factor (VEGF) receptor VEGFR1 (flt-1) and VEGFR2 (KDR) extracellular domains fused in series to the Fc portion of human IgG1. It acts by binding and inactivating VEGF in the circulation and in tissues. VEGF Trap has substantially greater (1-5 pM) affinity for the VEGF ligand than monoclonal antibodies. Preclinical studies indicate that subcutaneously (sc) administered VEGF Trap can substantially inhibit the growth of a variety of tumors implanted in mice. Preclinical pharmacokinetics predicted a half-life compatible with weekly dosing in humans. In this open-label, dose-escalation phase 1 study, a single sc dose of VEGF Trap is given to patients with relapsed and refractory solid tumors and lymphoma followed 4 weeks later by 6 weekly (sc) doses of the drug. Samples for pharmacokinetic analysis are collected both after the single dose and during chronic treatment. Patients are monitored for the development of anti-VEGF Trap antibodies. Anti-tumor efficacy is assessed by measuring changes in tumor mass clinically and/or by MRI. Tumor perfusion and water content is assessed in a subset of patients by dynamic contrast (Gadolinium)- enhanced MRI techniques. To date 6 patients have been treated on two dose levels: 25mcg/kg and 50mcg/kg. Early data reveals that the VEGF Trap complexes to circulating VEGF in plasma. To date, no anti-VEGF Trap antibodies have been detected in any of the patients treated. Longer term results for a larger number of patients and the pharmacokinetics of the Trap and Trap:VEGF complexes will be discussed.

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In vitro pharmacological profiles and *in vivo* anti-angiogenesis activity of S-3304, a novel matrix metalloproteinase inhibitor

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S-3304, a Na-[[2-[5-[[4-methylphenyl]ethynyl]thienyl]]sulfonyl]-D-tryptophan, which is synthesized through a few steps from commercially available compounds, is an orally-active and non-cytotoxic inhibitor of matrix metalloproteinase (MMP). The inhibitory effect of S-3304 against various human MMPs was examined in *in vitro* enzyme assay. S-3304 most potently inhibited the activities of MMP-2, -8, -9, -12, -13, weakly inhibits MMP-3-CD, -10, -14, -15, and -16, but does not inhibit MMP-1, -3 or -7. Crystal structure of DeltaFND-MMP-9 complexed with S-3304 was solved (Space group: P212121, Cell constants(Å): a=37.04, b=52.01, c=69.14,). The electron density map with 1.8 Å resolution revealed the interaction between S-3304 and the active site of the protein. In crystallographic data, it was clear that S-3304 sits in the S1' pocket deeply and nicely as a drug compound. We next examined the MMP inhibitory activity of S-3304 using gelatin zymography. The result showed that the gelatinase activity of MMP-2 and -9, derived from human tumor cells, was completely inhibited by S-3304. Furthermore, the effect of S-3304 on tumor-induced angiogenesis was investigated by the dorsal air-sac method. 107 cells of HT1080 human fibrosarcoma cells, which produce angiogenesis factors including VEGF, were filled into a chamber. The chamber was subcutaneously implanted into the dorsal side of mice. S-3304 was orally administered to the chamber-implanted mice twice a day at a dose of 20 and 200 mg/kg. Four days after implantation, the skin on the chamber was removed and fixed. The number and vascular area of vessels beneath the musculi cutaneous were histologically analyzed. Treatment with S-3304 resulted in reduction of the number and vascular area of vessels. Thus, S-3304 significantly inhibited the tumor-induced angiogenesis. All animal studies were approved by the Animal Care and Use Committee prior to initiation.

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Anti-angiogenic activity of the VEGF receptor tyrosine kinase inhibitor ZD6474

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ZD6474 is a small molecular weight inhibitor of KDR tyrosine kinase and a potent inhibitor of VEGF-induced human umbilical vein endothelial cell (HUVEC) proliferation (IC_{50} = 60 nM) that is in clinical development. Consistent with anti-angiogenic activity, the compound has demonstrated broad-spectrum activity in pre-clinical tumour models following chronic oral administration. ZD6474 has also been shown to inhibit ossification in the femoral growth plate of young rats; a physiological process which is dependent upon angiogenesis. For further confirmation of anti-angiogenic activity, ZD6474 was examined in two additional preclinical models. An *in vitro* model of endothelial cell tube formation was first used to determine the effect of ZD6474 on tubule growth and morphology. HUVEC and human fibroblasts were obtained as commercial co-cultures (AngioKit, TCS Cellworks, UK). Cells were maintained in MCDB131 media with or without ZD6474 for 11 days. To quantify tubule growth a novel whole-well method was developed using a Zeiss KS400 3.0 image analyser (Imaging Associates Ltd). Tubule formation was examined at day 11 following fixation and staining of tubules for CD31. Morphological parameters measured were total number of branch points, total tubule length and total area of tubule growth: ZD6474 inhibited each parameter significantly, with IC_{50} values of 33nM, 61nM and 93 nM respectively. An intradermal (i.d.) model of tumour-induced angiogenesis was then used to assess the effects of ZD6474 treatment *in vivo*. Male nude mice were implanted intradermally with A549 human lung tumour cells (1x107 cells/implant, 2 implant sites per mouse). Two additional injections of phosphate buffered saline (50 ul) were administered to each mouse as a control. ZD6474 (50 or 100mg/kg) or vehicle was administered orally for 5 days. Following treatment (day 6) the total number of blood vessels (major vessels and branching points) was determined within a 1cm^2 area around each implant site by light microscopy. A549 tumour cells induced significant angiogenesis, i.e. 152 $\pm$ 6.5 vessels compared with a background count of 27 $\pm$ 1.2 vessels in vehicle implants (mean $\pm$ S.E.). Treatment with 50 or 100 mg/kg/day ZD6474 inhibited the tumour-induced blood vessel formation by 63% and 79% respectively ($P<0.001$). These additional data are confirmatory of the anti-angiogenic activity of ZD6474.

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A phase I, double-blind, randomized, placebo-controlled study to investigate the safety tolerability and pharmacokinetic profile of S-3304, a matrix metalloproteinase inhibitor, when given in multiple doses with high doses for 4 weeks to healthy volunteers

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Objectives: This study was conducted to define maximum tolerated dose of S-3304 in normal healthy volunteers prior to initiation of a patient phase I study with solid tumors.
Study design: Eight subjects were randomized, 6 subjects to receive S-3304 and 2 subjects placebo at the following dose levels: 800 mg bid, 1600 mg bid, 2400 mg bid and 3200 mg bid. Subjects were to take study drug orally after meals once on Day 1, twice daily on Day 3 - 27 and once on Day 28. Safety assessment was based upon symptoms, signs, clinical laboratory tests and ECG. Dose escalation or study treatment was to stop, if three or more subjects at one dose level either: (1) experienced a Dose Limiting Toxicity defined as > grade 2 toxicity (NCI CTC); (2) had a hepatic transaminase of > 2.5 times upper limit of normal reference range; or (3) were withdrawn from further dosing due to symptoms interfering with normal daily activities. The protocol was approved by the local ethics committee prior to the study.
Results and Discussion: 4 male and 4 female subjects were enrolled to each dose group. All subjects at 800 mg bid completed the treatment. Two subjects were discontinued from treatment with 1600 mg bid, due to increased transaminases (grade 1 toxicity) and increased creatinine phosphokinase (grade 3 toxicity) respectively. One subject was discontinued from treatment at 2400 mg bid due to transient hair loss. Five subjects were withdrawn from treatment with 3200 mg bid due to: raised hepatic transam-

inases grade 2 toxicity (2 subjects) and symptoms including headache, blurred vision, abdominal pain and vomiting (3 subjects). One of these was randomized to placebo. In the 3200mg bid group, all subjects randomized to S-3304 had hepatic transaminases increased to grade 1 or 2. The correlation between plasma drug concentration and raised transaminases is yet to be determined. Only the minority of subjects reported mild myalgia/arthralgia symptoms, and this did not interfere with their normal daily activities. AUC0-12,ss and Cmax,ss of S-3304 increased with dose level but less than proportionately (see Table).

Table. Steady-State Pharmacokinetic (PK) parameters of S-3304 (mean ± SD)

Dose level	800 mg*	1600 mg*	2400 mg*	3200 mg**
$AUC_{0-12,ss}$ (μg*hr/mL)	411±117	466±80	634±170	943±317
C_{max} (μg/mL)	80±20	93±13	120±21	140±27
$T_{1/2}$ (hr)	14.2±1.2	14.8±3.4	15.9±1.8	ND

*Day 28; **Day 14 (Dose discontinued before Day 28); ND: not determined

Pharmacokinetic parameters of metabolites will also be analysed.

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Dose and schedule optimization of a novel anti-angiogenic/anti-metastatic peptide, ATN-161 (Ac-PHSCN-NH2), which targets multiple fully activated integrins including alpha-5 beta-1 and alpha-v beta-3

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ATN-161 (Ac-PHSCN-NH2) is currently completing pre-clinical development with the initiation of a phase I trial anticipated in October, 2002. Previously published data has demonstrated the ability of this peptide to inhibit tumorigenesis, angiogenesis and metastasis of subcutaneously inoculated tumors in a syngeneic (Mat LyLu) model of prostate cancer [Livant et al. (2000) Cancer Res 60: 309]. In addition, ATN-161 has been shown to inhibit angiogenesis in liver metastasis from intrasplenically injected CT26 mouse carcinoma cells [Stoelzing et al., Clin Cancer Res (2001) 7: 3656s]. We have extended these results to a syngeneic Lewis Lung Carcinoma (3LL) model and have observed that ATN-161 inhibits tumor growth as effectively as metronomically administered cyclophosphamide (170 mg/kg q6d) in the early 3LL model (ATN-161 given on or before day 6 after tumor cell inoculation). We have used this 3LL model to optimize dose as well as schedule. The inhibition of 3LL tumor growth by ATN-161 observed a U-shaped dose response with 1-10 mg/kg being the optimal dose. No anti-tumor effects were observed at 0.2 mg/kg and very little effect was observed at 100 mg/kg, the highest non-toxic dose tested, with escalating anti-tumor effects observed at 50, 25 and 12.5 mg/kg, respectively. This U-shaped dose response was confirmed using the CT26 metastasis model. At the optimal dose of ATN-161 (1 mg/kg), significant inhibition of liver metastasis was observed whereas no activity was observed at the high dose of ATN-161 (100 mg/kg) tested. Schedule optimization was also evaluated using the 3LL model. The optimal schedule was determined to be ATN-161 (1 mg/kg) q3d. These results will provide rationale for the starting dose and schedule for phase I trials.

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In vitro antiangiogenic activity of thalidomide analogues

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Thalidomide, also known as (alpha-(N-phthalimido)-glutarimide), is currently in Phase II clinical testing as a single agent or in combination with chemotherapy against a number of solid tumors such as gliomas, prostate and renal cell carcinomas. The resurgence of interest in thalidomide can be attributed to its antiangiogenic activity, which was shown to be mediated by a metabolilte – 5'OH thalidomide. Using the backbone of the metabolite, we synthesized 118 unique thalidomide analogues and examined their antiangiogenic activity *in vitro*. Preliminary experimental data selected seven of these analogues for further evaluation. In the rat aortic ring assay, six of the seven analogues significantly inhibited microvessel outgrowth at 12.5-200 uM. Thalidomide failed to block angiogenesis at similar concentrations. Subsequently, the effects of these analogues on human umbilical vein endothelial cell (HUVEC) proliferation and tube formation were studied. Six of the seven analogues demonstrated antiproliferative action in HUVECs. Cell proliferation was not affected by thalidomide. Interestingly, all seven analogues as well as thalidomide suppressed tube formation. Analogues in the tetrafluorophthalimido class showed the highest potency and efficacy in all three assays. Taken together, our results support the further development of thalidomide analogues as antiangiogenic agents. The *in vivo* toxicology and therapeutic potential of the described analogues in the treatment of prostate cancer are presently under investigation.

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VEGF-Trap: a novel, potent VEGF blocker with anti-tumor effects

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Vascular endothelial growth factor (VEGF) plays a critical role during the normal process of angiogenesis required for embryonic development, and plays a key role in the pathological angiogenesis that occurs in a number of diseases, including cancer. One of the most effective ways to block the VEGF-signaling pathway is to prevent VEGF from binding to its normal receptors by administering decoy soluble receptors. By determining the requirements to maintain high affinity while extending *in vivo* half-life, we were able to engineer a very potent VEGF blocker with desirable pharmacokinetic properties. The resulting VEGF Trap, a soluble decoy receptor created by fusing the ligand-binding immunoglobulin domains of VEGF receptor 1 (VEGFR1) and VEGF receptor 2 (VEGFR2) to the constant region (Fc) of human IgG1, is the highest affinity VEGF blocker described to date, with an affinity for VEGF of 1-5 pM. The VEGF-Trap effectively suppresses tumor growth and vascularization *in vivo*, resulting in stunted, and almost completely avascular tumors. VEGF Trap mediated blockade may be superior to that achieved by other agents, such as monoclonal antibodies targeted against the VEGF receptor. The VEGF Trap is currently undergoing a Phase I clinical trial.

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Tumor genotype, RRM1 expression, and outcome of patients with lung cancer

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We have described frequent allele loss on chromosome segment 11p15.5 and its association with metastatic spread and shortened survival in patients with non-small cell lung cancer. Patients with stage I disease, i.e. absence of spread to lymph nodes, and allele loss had survival comparable to patients with stage II disease, i.e. cancer present in lymph nodes. We have recently reported the complete genomic sequence and transcript map for the minimal region of allele loss, and it encompasses the complete gene for the regulatory subunit of ribonucleotide reductase (RRM1). However, functional inactivating mutations were not found by screening a subset of NSCLC. Our recent cell biological studies have provided evidence for a functional role of RRM1 in suppression of cell migration, invasion, and *in vivo* metastasis formation, that is independent of an alteration in the deoxynucleotide (dNTP) pool. Other investigators have reported reduced anchorage independent growth in ras-transformed mouse fibroblasts transfected with RRM1 and a role for RRM1 in microtubule nucleation of centromeres in Xenopus. Here, we measured the level of expression of RRM1, compared to the expression of RRM2 (catalytic subunit of ribonucleotide reductase) and p53R2 (catalytic subunit involved in dNTP supply for DNA damage repair), and investigated the association with allele loss, RRM1 promoter polymorphisms, and survival. Tissue specimens from 51 patients undergoing resection for NSCLC were collected and immediately frozen in liquid nitrogen. Total RNA was extracted, reverse transcribed, and used for real-time quantitative PCR (ABI Prism 7700). Primers and probes for the genes RRM1, RRM2, and p53R2 were designed to cross introns, and the amplicons were 95 bp, 90 bp, and 105 bp respectively. Gene expression was normalized using 18S rRNA as reference. We found that RRM1 expression was associated with RRM1/D11S4932 allele loss, with a median RRM1 value of 3.8 in specimens with allele loss compared to 43.7 in those without allele loss. RRM1 expression was also associated with the A/C promoter polymorphism, with a median RRM1 value of 12.9 in patients with the CC allelotype, 72.8 in those with the AA allelotype, and 22.8 in heterozygotes (AC allelotype). RRM1 and RRM2 expression were highly correlated