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PEGylated DX-1000: pharmacokinetics, anti-tumor and anti-metastatic effects of a specific plasmin inhibitor

L. Devy[1], S. Rabbani[2], L. Naa[1], J. Chen[3], M. Toews[3], M. Stochl[3], A. Ley[3], R.C. Ladner[3], D.T. Dransfield[3], P. Henderikx[1]. *[1]Dyax s.a., Cell Biology, Liege 1, Belgium; [2]Department of Medicine and Oncology, McGill University Helath Centre, Montreal, Qc, Canada; [3]Dyax corp., Cambridge, MA, USA*

Plasmin is a serine protease predominantly present in the body in its inactive zymogen form, plasminogen. In tumors, activation of plasminogen mainly occurs locally by urokinase (uPA) overproduced by cancer or stromal cells. Using phage display, we have identified a TFPI-derivative Kunitz domain protein, which is a specific inhibitor of plasmin (Ki = 88pM) referred to as DX-1000. DX-1000 has been produced in Pichia pastoris. DX-1000 was tested in several functional cell-based activity assays and demonstrated potent inhibitory activity. DX-1000 specifically blocked plasmin-mediated proMMP-9 activation in tumor cells. When evaluated in vitro in a chemoinvasion assay, DX-1000 efficiently inhibited the invasiveness of uPA-expressing HT-1080 cells (cell invasion reduced by 46% and 66% with 1 and 100 nM DX-1000, respectively). We demonstrated that DX-1000 was able to efficiently inhibit tube formation of HUVECs (IC50 = 1.39±0.28 nM) and mouse endothelial cells (IC50 = 16.6±0.1 nM) highlighting the usefulness of this plasmin inhibitor as an effective inhibitor of angiogenesis. DX-1000 holds great promise as a candidate for treating cancer. However, due to its low molecular weight (7 kDa), the protein exhibits a rapid plasma clearance rate in vivo (β Phase half-life ~27 minutes in mice and 1 hour in rabbits) thereby limiting its therapeutic effectiveness. PEGylation overcomes these shortcomings. By increasing the molecular mass of DX-1000 from 7 to 27 kDa (5k PEG added at 4 sites), we dramatically improved pharmacokinetics of DX-1000. PEGylation of DX-1000 substantially prolongs in vivo circulation and stability (β Phase half-life ~13 hours in mice and 59 hours in rabbits) while having no effect on the activity or potency of DX-1000 as a plasmin inhibitor in several in vitro cell-based assays. Interestingly, PEGylated DX-1000 significantly reduced primary tumor progression by 45% and reduced liver and lung metastasis by 40% in a MDA-MB-231 orthotopic model in mice. Studies are ongoing to elucidate the cellular mechanism involved in the observed anti-tumor and anti-metastatic activities. In conclusion, we have generated a longer half-life version (through PEGylation) of our potent plasmin inhibitor without altering in vitro activity and demonstrating in vivo anti-tumor and anti-metastatic activities.

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The effects of endothelial cells on tumor cell gene expressions

T. Kaneko[1,2], H. Suda[2], J.E. Nör[1]. *[1]University of Michigan, Department of Restorative Sciences, Michigan, USA; [2]Tokyo Medical and Dental University, Department of Restorative Sciences, Tokyo, Japan*

Background: The effect of tumor cell derived factors on angiogenesis has been well established. In contrast, little is known about the role of factors secreted by endothelial cells on tumor cell phenotype. The role of endothelial cells in tumor biology has been considered primarily lining the vessels required for the influx of oxygen and nutrients to tumor cells. To know the effect of endothelial cells on tumor cell gene expressions, we performed a co-culture system between endothelial cells and tumour cells.
Material and Methods: Human dermal microvascular endothelial cells (HDMEC; Clonetics) stably transduced with Bcl-2 (HDMEC-Bcl-2), empty vector controls (HDMEC-LXSN), and Human squamous cell carcinoma cell lines, UM-SCC-17B and OSCC3, were used. When indicated, endothelial cells (HDMEC) were cultured in the lower chamber, and tumor cells (UM-SCC-17B or OSCC3) in the upper chamber of a non-contact co-culture system for 24−48 h. These cells were separated by the 1 μm pore membrane of transfer wells. We cultured cells in 1 μg/ml polyclonal anti-human VEGF, or 21 μg/ml polyclonal anti-human VEGFR1 (Flt-1) antibody to neutralize the activity of these signaling molecules, and measured VEGF protein expression by ELISA. Total RNA was extracted from each cell line and Bcl-2, CXCL8, CXCL1 expression levels were examined by RT-PCR or real time PCR.
Results: Bcl-2, CXCL8 and CXCL1 mRNA expressions in tumour cells were upregulated when these cells were co-cultured with HDMEC-Bcl-2 as compared to co-culture with empty vector HDMEC-LXSN. Both squamous cell carcinomas studied here (UM-SCC-17B and OSCC3) expressed VEGFR1, and Bcl-2 mediated the upregulation of VEGF expression in endothelial cells. Blockade of the VEGF signalling pathway with a neutralizing antibody inhibited the ability of HDMEC-Bcl-2 to induce Bcl-2, CXCL8 and CXCL1 expressions in UM-SCC-17B cells. Likewise, blockade of VEGFR1 prevented upregulation of Bcl -2, CXCL8 and CXCL1 expressions in the tumour cells co-cultured with HDMEC-Bcl-2.
Conclusions: These data suggested that the endothelial cells may secrete factor(s) that directly influence gene expression levels in the tumour cells. These data also demonstrate that VEGF signalling through VEGFR1 is a key event in the process through which endothelial cells modulate gene expression in tumour cells.

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A phase I study examining weekly weight based or fixed dosing and pharmacokinetics (PK) of a novel spectrum kinase inhibitor, XL999, in patients (pts) with advanced solid malignancies (ASM)

M. Mita[1], J. Cooper[1], A. Ricart[1], A. Mita[1], C. Takimoto[1], S. Britt[1], A. Tolcher[1], A. Dowlati[1], K. Papadopoulos[1]. *[1]Cancer Therapy And Research Center, Institute For Drug Development, San Antonio, USA; [2]Case Western Reserve University, Cleveland, Ohio, USA*

Background: XL999 is a small molecule inhibitor of multiple kinases involved in tumor cell growth, angiogenesis, and metastasis, including VEGFR2 (KDR), PDGFRα/β, FGFR1/3, FLT-3, and SRC. A phase I study of XL999 administered as a 4 hr infusion every 2 weeks in pts with ASM showed that the maximum tolerated dose (MTD) was 3.2 mg/kg and the plasma $t_{1/2}$ was approximately 24 hrs. Three pts dosed at 0.2–1.6 mg/kg had partial responses and 9 pts had stable disease (SD) >3 months. Based on these data, a weekly (wkly) weight based and fixed dosing schedule with PK monitoring was further explored.
Methods: XL999 at 2.4 mg/kg or 200 mg was administered to pts as a 4 hr infusion on day (d) 1 and d8 with toxicity assessment. PK sampling was performed on d1 & d15. Pts received further doses of XL999 wkly in the absence of unacceptable toxicity, disease progression, or accumulation based on PK results.
Results: As of June 1, 2006, 12 pts were enrolled and received XL999 weekly as initial treatment at 2.4 mg/kg or 200 mg fixed dose. Of 8 pts treated at 2.4 mg/kg, none had experienced any G2 or worse drug related adverse events. One asymptomatic pt at 2.4 mg/kg with non-specific ECG changes after d1 was discontinued from study. One pt receiving 200 mg experienced symptomatic hypotension and non-specific ECG changes on d1 and was discontinued. Four of 8 evaluable pts had SD for >2 months. In patients with complete d1 & d15 PK at 2.4 mg/kg (n = 5) or 200 mg (n = 4), there was moderate interpatient and intrapatient variability, with no evidence of drug accumulation on repeat dosing.

Averages for weekly dosing PK parameters[a]

Dose level	Cycle	t 1/2 (hr)	Cmax (ng/mL)	AUC_{0-inf} pred (hr ng/mL)	CL pred (L/hr)
2.4 mg/kg	1	20.8 (16%)	513 (34%)	5939 (45%)	40.9 (36%)
	2	23.2 (28%)	670 (41%)	7359 (35%)	32.8 (41%)
200 mg	1	36.1 (54%)	528 (44%)	7737 (52%)	32.1 (51%)
	2	29.2 (11%)	491 (42%)	6476 (44%)	34.6 (32%)

[a]%CV in parentheses.

Conclusions: XL999 administered wkly as a 4 hr infusion at a dose of 2.4 mg/kg or 200 mg appears to be well tolerated with no evidence of drug accumulation. Drug clearance appears independent of weight and supports the use of fixed dosing.

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Preclinical studies in a murine tumour model on the efficacy of combining radiation with angiogenesis inhibitors and vascular disrupting agents

M. Horsman, R. Murata. *Aarhus University Hospital, Dept. Experimental Clinical Oncology, Aarhus, Denmark*

Background: Targeting tumour vasculature is becoming an increasing popular therapy, but the ability of angiogenesis inhibitors (AIAs) and/or vascular disrupting agents (VDAs) to inhibit tumour growth is limited. As such, these vascular targeting agents (VTAs) are now being combined with other therapies, (i.e., radiation). The aim of this study was to investigate the potential of combining both AIAs and VDAs with radiation.
Materials and Methods: A C3H mammary carcinoma grown in the right rear foot of female CDF1 mice was used in all experiments. Treatments were performed when tumours had reached 200 mm^3 in size. The AIA and VDA were TNP-470 (Takeda Chemical Industries) and combretastatin A-4 disodium phosphate (CA4DP; OXiGENE, Inc.),

respectively. Both were prepared in saline immediately before each experiment and given either as a single intraperitoneal injection (CA4DP; 250 mg/kg), or subcutaneously every second day for two weeks (TNP-470; 100 mg/kg). Radiation (1×10 Gy; 240 kV x-rays) was administered locally to the tumour bearing foot of restrained non-anaesthetised mice. Response to treatment involved measuring tumour volume 5 times/week and calculating the tumour growth time (TGT; time to reach 3 times the treatment volume). Statistical analysis was performed using the Student's t-test, with the significance level being $p < 0.05$.

Results: The mean (±SE) TGT for untreated control tumours was 4.4 days (4.1–4.7). This TGT was increased to 5.5 (5.1–5.9), 5.5 (4.8–6.2) and 6.0 (5.4–6.6) days, by CA4DP, TNP-470 and CA4DP + TNP-470, respectively. However, only in the CA4DP treated groups were these increases significant. A significant increase to 12.6 days (12.0−13.2) was found following irradiation. Injecting CA4DP within 1-hour after irradiating non-significantly increased the radiation TGT to 14.4 days (13.1−15.7), but a further significant increase was observed when radiation was given with either TNP-470 alone or the combination of CA4DP + TNP-470; the respective TGTs being 36.2 days (33.6−38.8) and 50.3 days (46.1−54.5). This response to TNP-470 + CA4DP with radiation was significantly greater than that for TNP-470 and radiation. Additional studies are ongoing to produce full radiation dose response curves with each combination treatment.

Conclusions: VTAs had very little effect on the growth of this C3H mouse mammary carcinoma when used alone or in combination. However, they significantly improved the tumour response to radiation. The greatest affect was obtained when both the AIA and VDA were combined, resulting in a 4-fold increase in radiation response.

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Inhibition of tumor cell invasion by a heparanase inhibitor and expression of a dominant negative mutant of heparanase

S. Simizu[1], K. Ishida[1,2], H. Osada[1]. [1]*RIKEN, Antibiotics Laboratory, Wako, Japan;* [2]*Taiho Pharmaceutical Co., Ltd., Hanno, Japan*

Background: Overexpression of heparanase has been observed in many human tumors, such as head and neck tumors, suggesting an involvement of heparanase in tumor progression. Thus, heparanase may be considered as a molecular target for the development of cancer therapy. We have reported some heparanase inhibitors, including RK-682, which inhibited tumor cell migration and invasion. In this report, we demonstrated that treatment with a heparanase inhibitor, 4-Bn-RK-682 and expression of an active site-deficient heparanase (HP/E225A) suppressed tumor cell invasion.

Materials and Methods: Homology Modeling of Heparanase: The amino acid sequence alignment of human heparanase and 1,4-beta-xylanase from *Penicillium simplicissimum* was carried out manually using the Homology module of the Discover/Insight II Programs.

Establishment of Heparanase-overexpressing Stable Cell Lines: We established the clones expressing high levels of heparanase protein or HP/E225A protein were designated HT1080-HP cells and HT1080-HP/E225A, respectively.

Results: To develop selective heparanase inhibitors, we synthesized several RK-682 derivatives based on the rational drug design. Among them, 4-Bn-RK-682 has been found to possess a selective inhibitory activity for heparanase. 4-Bn-RK-682 also inhibited the invasion and migration of HT1080 cells. To evaluate antimetastatic potencial of 4-Bn-RK-682 *in vivo*, 4-Bn-RK-682 (50 mg/kg) was administered p.o. in mice received B16ML6 melanoma cells intravenously. After 15 days, about 40% of the number of B16BL6 metastases in their lungs were suppressed.

Moreover, we found that overexpression of heparanase stimulated tumor cell migration, on the other hand, expression of HP/E225A reduced the migration of HT1080 cells. The sample incubated with the extract from HT1080-HP/E225A cells possessed weaker heparanase activity than that from HT1080-Neo cells, indicating that expression of HP/E225A protein suppressed endogenous heparanase activity. Furthermore, the amount of cell surface HS level was dramatically increased in HP/E225A-expressing cells. Therefore, it is suggested that HP/E225A functioned as dominant negative manner, thereby suppressing tumor cell migration.

Conclusions: Strategies that inhibition of heparanase by treatment with heparanase inhibitor and gene transfer of HP/E225A may be effective therapies for those human cancers that are expressing heparanase.

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Additive action of a novel Cathepsin K inhibitor and zoledronic acid (Zometa) in a model of osteolytic human breast cancer metastasis

M. Waltham[1,2], N. Sims[1,3], E. Williams[4], A. Connor[1,2], T. Kalebic[5], J. Zimmerman[6], E. Thompson[1,2,3]. [1]*St. Vincent's Institute, Melbourne, Australia;* [2]*University of Melbourne, Department of Surgery, Melbourne, Australia;* [3]*University of Melbourne, Department of Medicine, Melbourne, Australia;* [4]*Bernard O'Brien Institute for Microsurgery, Melbourne, Australia;* [5]*Novartis Pharma, Oncology Research, New Jersey, USA;* [6]*Novartis Pharma, Oncology Research, Basel, Switzerland*

Bisphosphonates are a class of drugs that inhibit the breakdown of bone, and are proving useful in the management of breast cancer patients with bone metastasis, where tumour cells activate osteoclasts to degrade bone. However, many breast-bone metastases do not completely benefit from bisphosphonate therapies, and there is very little impact on overall survival, emphasizing the great need for additional therapies. Cathepsin K is a cysteine peptidase secreted by osteoclasts that degrades collagen in the acidic lacunal space, and thus contributes to the bone destruction associated with bone metastasis.

Our study was designed to examine whether a newly derived Cathepsin K inhibitor from Novartis (CK-1) may work synergistically in conjunction with bisphosphonate. Female SCID mice were inoculated intratibially with T47D human breast cancer cells, and treated with a single i.v. bolus of bisphosphonate (zoledronic acid [Zometa]: 10, 50 or 100 ug/kg) two weeks after inoculation. Mice were then randomised to additionally receive either CK-1 (50 mg/kg) or vehicle i.p. twice daily from two weeks post inoculation, to provide combinations of each dose of Zometa with or without CK-1 ($n = 8$ per group).

Although only a slight inhibition of tumour-induced bone degradation was observed with the highest dose of bisphosphonate at 7 weeks post-inoculation, co-treatment with cathepsin K inhibitor significantly reduced bone loss ($p = 0.0472$ compared to Zometa alone).

These data clearly demonstrate the potential of CK-1 to complement bisphosphonate treatment of lytic bone metastasis associated with breast cancer.

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Ascorbate (vitamin C): friend or foe in the fight against cancer

S. Telang, A. Yalcin, A. Clem, J. Eaton, J. Chesney. *University of Louisville, Louisville, USA*

Ascorbic acid (Vitamin C, AA) is an essential dietary factor for humans and other primates due to a genetic absence of the key synthetic enzyme l-gulono-γ-lactone oxidase. The role of AA in enhancing immunity against cancer is widely accepted, although the precise mechanisms of this effect are not well understood. Recently, AA was identified as a cofactor in the hydroxylation and subsequent targeting for proteolysis of Hypoxia Inducible Factor-1α, a transcriptional regulator of the neoplastic response to hypoxia. The importance of AA in augmenting immunity and HIF-1 degradation has led several investigators to conclude that physiological or mega-physiological plasma levels should suppress tumor growth. AA however may also be critical for angiogenesis which is a prerequisite for tumor growth *in vivo*. Effective angiogenesis requires the deposition of type IV collagen into the basement membrane of blood vessels by endothelial cells. Type IV collagen production is dependent on the hydroxylation of proline by prolyl hydroxylase which requires AA as a co-factor.

We hypothesize that the requirement of AA for angiogenesis supercedes the requirements for anti-tumor immunity and HIF-1 degradation, and that dietary AA restriction will cause decreased angiogenesis and tumor growth. We measured type IV collagen produced by human umbilical vein endothelial cells (HUVECs) exposed to 0–200 μM AA (physiological [AA] = 50–100 μM) and found that a minimum of 25 μM AA is needed for immunoreactive type IV collagen production. We surmised that decreased type IV collagen deposition by HUVECs would cause poor tube formation on Matrigel *in vitro*. We observed intact tubes in 50–100 μM AA but disorganized tubes at lower (0−25 μM) and higher (200 μM) [AA]. Mice containing a homozygous genomic deletion for the last enzyme in AA synthesis l-gulono-γ-lactone oxidase (Gulo −/−) die of scurvy within 50 days of dietary AA restriction. We implanted Lewis lung carcinoma cells s.c. into Gulo −/− mice depleted of AA for 28 days, and then continued to restrict (0 mg/day) or fully supplemented the mice with AA (1.6 mg/day). After 12 days, we observed markedly decreased tumor growth in restricted mice (tumor mass: 1124±208.6 mm^3 [1.6 mg/day]; 231±68 mm^3 [0 mg/day] *p-value* <0.005). Microscopy of tumor sections from AA restricted mice showed greatly reduced capillaries (5±2/HPF) compared with repleted mice (19±3/HPF) and, in addition, substantially less collagen staining. Surprisingly, we found no significant differences in