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Pre-clinical evaluation of the novel alkylating agent RH1 against paediatric tumour cell lines

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Despite dramatic improvements in survival from childhood cancer there remain many tumour types in which drug resistance is a major problem. There is thus an urgent need for access to novel agents for this group of patients. RH1 (2,5-diaziridinyl-3-[hydroxymethyl]-6-methyl-1,4-benzoquinone) is a novel alkylating agent which is currently in phase I trial in adults. The bioreductive pro-drug is activated to a more potent DNA interstrand cross-linking moiety by the obligate two-electron reductase DT-diaphorase (DTD), which is widely expressed in tumour cells. In keeping with its mechanism of action human colon and lung carcinoma cells that over-express DTD are more sensitive to RH1.

We evaluated the efficacy of RH1 against a range of paediatric tumour cells lines in vitro. DTD protein expression in a panel of childhood cancer cell lines was measured by Western blotting. A high and a low DTD expressing cell line from each of neuroblastoma, osteosarcoma, and Ewing's sarcoma was chosen to investigate further. Comparison of the cytotoxicity of RH-1 to that of cisplatin and doxorubicin using long term clonogenic assays showed IC50 values ranging from 1.5–7.5 nM, even in cell lines relatively resistant to cisplatin and doxorubicin. There was no correlation between sensitivity and DTD expression. However in short term SRB assays IC50 doses ranged from 1–200 nM and, as expected, sensitivity correlated with DTD expression. Finally, the combination index equation was used to define synergistic interactions between RH1, cisplatin and doxorubicin. Preliminary results suggest that in at least three cell lines, RH1 is synergistic with doxorubicin or cisplatin.

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Metabolism and pharmacokinetics of PR-104, a hypoxia-activated nitrogen mustard prodrug in phase I clinical trial

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PR-104 is a soluble phosphate ester "pre-prodrug" designed to be converted *in vivo* to the corresponding alcohol PR-104A, which is a hypoxia-activated nitrogen mustard prodrug. PR-104 shows strong antitumour activity in human tumour xenograft models, and is currently in Phase I clinical trial through Proacta Inc. Here we report initial results of a drug metabolism/pharmacokinetics (PK) study of PR-104.

The plasma PK of PR-104 and PR-104A was evaluated in three preclinical species (CD-1 nude mice, Sprague Dawley rats, and beagle dogs) at a range of doses, and in the initial cohorts of the Phase I trial, by HPLC/mass spectrometry with tetra-deuterated internal standards of both analytes.

PR-104 was rapidly hydrolysed to PR-104A in all species. A two compartment PK model, in which PR-104 is converted to PR-104A in the central compartment, fitted the plasma PK of both analytes. The model parameters were similar across species, with the exception of faster clearance of PR-104A in dogs (terminal half life 10 vs 17, 19 and 22 min in mice, rats and humans). No difference was seen between genders, and PK was little changed on the last dose of a qwx4 schedule in rats and dogs. The plasma PK of both analytes showed slight non-linearity with dose, which could be accounted for by a minor saturable elimination pathway for PR-104 itself.

An excretion and tissue biodistribution study in CD-1 nude mice bearing SiHa tumors, using [3]H-PR-104, demonstrated almost quantitative elimination within 48 hr (urine 46.3±1.8% [SEM] of total dose, faeces 50.2±2.4%). Retained radioactivity at 48 hr was highest in tumour and lowest in brain. The metabolite profile in plasma and urine was characterized by capillary LC/ion trap MS. Products of the mercapturic acid pathway were prominent in all species (especially a cysteine adduct of PR-104A), along with oxidative debromoethylation to the mesylate half mustard (especially in rodents) and *O*-glucuronidation of the alcohol side chain in dogs and humans.

Overall, this study demonstrates that the plasma PK and metabolism of PR-104 is similar in humans and preclinical species. Rapid hydrolysis of the phosphate to the corresponding alcohol competes effectively with its urinary elimination. PR-104A is extensively metabolised by GSH conjugation and glucuronidation, and by oxidative metabolism of the mustard moiety. The reduced metabolites responsible for metabolic activation in hypoxic tumour cells were not detected in plasma or urine.

Cellular therapies and cytokines

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Denileukin diftitox depletes T regulatory cells and causes regression of melanoma metastases in humans

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Background: Cognate immunity targeted against neoplastic cells depends on a balance between activated antigen-specific T cells and suppressive or regulatory T cells. Recently, a subset of CD4+CD25^{high} regulatory T (Treg) cells has been found to directly suppress the activation of anti-tumor effector T cells in a contact-dependent manner. Depletion of these Treg cells using anti-CD25 monoclonal antibodies induces a CD8+ T cell dependent immune rejection of melanoma in mice. Recombinant interleukin 2/diphtheria toxin conjugate (DAB[389]IL2) previously has been developed as a treatment for cutaneous CD25+ T cell lymphoma. DAB[389]IL2 binds to surface CD25 and, after internalization causes cell death within hours. We hypothesize that DAB[389]IL2 will selectively deplete CD4+CD25^{high} Tregs in patients with melanoma and allow induction of melanoma-specific immunity.

Materials and Methods: The effect of DAB(389)IL2 on tumor growth was examined in seven patients with Stage IV melanoma. DAB(389)IL2 (9 or 12 mcg/kg) was administered daily × 4 days every three weeks for four cycles. FDG-PET and/or CT imaging was obtained just prior to DAB(389)IL2 administration and within two weeks after completion of the fourth cycle. In a subset of patients, peripheral blood Treg cells were quantitated by flow cytometry (CD4+/CD25+/foxp3+) before and after DAB[389]IL2 administration. Immunohistochemical analyses of subcutaneous melanoma metastases were also performed for the melanoma-specific protein S-100 and the T cell surface antigen CD8.

Results: Two patients received 9 mcg/kg DAB(389)IL2 and, after two cycles, experienced overt progression consisting of a combination of tumor growth and newly detectable tumors. Five patients received 12 mcg/kg DAB(389)IL2 and, after four cycles, experienced significant regression of several metastatic tumors, including subcutaneous tumors and metastases in the liver and axillary lymph nodes. Two subcutaneous tumors in the lower extremity of a single patient became necrotic and infected, requiring surgical resection. Immunohistochemical analysis of these tumors revealed an apoptotic tumor surrounded by CD8+ T lymphocytes. The peripheral blood Treg concentration in this patient decreased after the second DAB[389]IL2 administration (day 1, 36.59 Tregs/microliter; day 7, 17.48 Tregs/microliter).

Conclusions: We conclude that depletion of Treg cells in tumor-bearing humans may allow the activation of cognate immunity leading to CD8+ T cell-mediated death of neoplastic cells. A phase II clinical trial to examine the efficacy of DAB(389)IL2 in this patient population that incorporates immunocorrelative analyses of peripheral blood and tumor-associated Treg cells and CD8+ melanoma-specific effector T cells is now underway.

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Recombinant human Interleukin-21 (rIL-21), a new cytokine for immunotherapy: results of two phase 1 studies in patients with metastatic melanoma (MM) or renal cell carcinoma (RCC)

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Background: rIL-21 is a pleiotropic class I cytokine that activates CD8+ T cells and NK cells. Based on preclinical data suggesting rIL-21 will have anti-tumor effects, clinical trials to characterize the safety and activity of this cytokine were initiated.

Methods: rIL-21 was administered by i.v. bolus injection to patients (pts) with AJCC stage IV MM or RCC in two phase 1 studies conducted in Australia or US. In the AUS study, two 6-week treatment regimens were tested during dose escalation: 6 cycles of thrice weekly dosing (3/w) or 3 cycles of 5 daily doses each followed by 9 days of rest (5+9). In the US study, a regimen of 2 cycles of 5 daily doses followed by 9 or16 days of rest was tested in two parts: a dose escalation part followed by a dose expansion part. Objectives were to estimate the maximally tolerated dose (MTD) and to assess pharmacokinetics, immunogenicity, immunomodulatory and anti-tumor activity of rIL-21.

Results: rIL-21 was administered to 44 pts at doses from 1–100 μg/kg during dose escalation and to 28 pts at 30 μg/kg during dose expansion.

22 pts received up to 11 cycles of extended treatment. The safety profile of rIL-21 was similar in the 2 studies and 2 patient populations. rIL-21 was well tolerated at doses of 1–10 μg/kg. Overall, the most commonly reported adverse events were fatigue, pyrexia, chills, nausea, and rash. In the 3/w regimen 30 μg/kg was declared the MTD based on DLTs in 3/7 pts and no higher doses were studied. The MTD for the 5+9 regimen was estimated to be 30 μg/kg in both studies though higher doses of 50 and 100 μg/kg were tolerated by some pts. Immunomodulatory effects were observed at all dose levels with increased levels of phosphorylated STAT3 even at the 1 μg/kg dose level; increased soluble CD25; increases in NK, CD8+ and CD4+ cells; and upregulation of perforin and granzyme-A & -B mRNA at doses ⩾3 μg/kg. Three pts (all RCC) achieved confirmed PRs and two pts (both MM, one previously treated in a vaccine study) achieved CRs according to RECIST after up to 11 cycles of treatment.
Conclusions: Based on data from 72 pts with MM or RCC exposed to rIL-21 in two phase 1 studies, rIL-21 was generally well tolerated. Relevant biological activity was observed at doses as low as 1 μg/kg. Four responses of 50 evaluable pts treated at doses ⩾30 μg/kg provide encouragement for future studies of rIL-21.

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Preclinical evaluation of IL-21 combination therapy with sorafenib and sunitinib in renal cell carcinoma

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Background: Sorafenib and sunitinib are tyrosine kinase inhibtors (TKIs) recently approved for the treatment of advanced RCC. Mechanisms of TKI-mediated tumor inhibition include direct inhibition of tumor cell proliferation and inhibition of angiogenesis via VEGF and PDGF pathways. While both drugs have demonstrated meaningful clinical benefit in RCC, most patients will relapse. The efficacy of TKIs may be improved in combination with other agents. IL-21 is a novel cytokine that has shown potent efficacy in preclinical models of RCC through mechanisms involving activation of NK cells and tumor-specific CD8 T cells. A Phase 1 study showed IL-21 to be tolerated as an outpatient regimen in RCC and pharmacologically active. Combining IL-21 with TKIs may result in greater clinical benefit by influencing multiple independent pathways. A series of preclinical pharmacology studies were designed with the objective of characterizing the potential pharmacologic interactions of TKIs and IL-21.
Methods: The effects of sorafenib and sunitinib on IL-21-mediated effector functions were tested under conditions of concurrent or sequential exposure using a range of concentrations of TKIs including steady state and maximal levels reported in patients. Assays employed measured NK cell cytotoxicity and IFNγ production, and IL-21 co-stimulation of CTL proliferation. Furthermore, effects of TKIs on IL-21R expression and STAT3 phosphorylation on PBMCs were evaluated. In vivo studies with murine IL-21 and TKIs were performed in subcutaneous B16 melanoma and RenCa RCC in mice.
Results: At steady-state concentrations of drug reported in serum of human patients, neither sorafenib nor sunitinib inhibited IL-21R expression or IL-21-induced STAT3 phosphorylation in human PBMCs, human or mouse CD4 and CD8 T cell proliferation, human NK cell granzyme B expression and ADCC activity. IL-21 treatment did not affect sunitinib or sorafenib-mediated anti-tumor effects in the syngeneic tumor models at maximal doses of TKIs. Additive anti-tumor effects were observed with IL-21 in combination with sub-maximal sorafenib.
Conclusions: Preclinical evaluation of the combination of TKIs and IL-21 suggests that the TKIs, when used at concentrations simulating therapeutic exposure, do not inhibit IL-21 or immune effector functions in vitro. Further, IL-21, in combination with TKI has additive effects in preclinical models, suggesting that testing of IL-21 and TKIs clinically is warranted.

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A chimera of interleukin-2 and a variant of the channel-forming protein aerolysin is selectively toxic to cells displaying the interleukin-2 receptor

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Background: Proaerolysin is the inactive precursor of the bacterial toxin aerolysin. The protoxin binds to GPI-anchored proteins (GPI-AP) on mammalian cells and is converted to aerolysin by proteolytic nicking. Aerolysin then spontaneously forms a stable oligomer that inserts into the plasma membrane and forms channels that cause cell death. Once inserted into the membrane, the toxin cannot leave, so that bystander cells cannot be affected: thus aerolysin may provide an advantage over enzyme toxins such as diphtheria toxin and exotoxin A, as a component of hybrid molecules that can target specific cell types.
Methods: To prevent binding of proaerolysin to normal cells, we made the variant R336A-PA. The R336 residue was identified based on our previous studies of proaerolysin binding to GPI-anchored proteins and the known structure of the protoxin. We also made a hybrid (IL-2-PA) of IL-2 fused to native proaerolysin and a second hybrid (IL-2-R336A-PA), by fusing IL-2 to R336A-PA. A six amino acid spacer separated the IL-2 and the proaerolysin. We determined whether the proaerolysin forms of these molecules could be converted to the aerolysin forms by proteolytic nicking, as well as the ability of the aerolysin forms to produce stable oligomers. Flow cytometry was used to compare binding of R336A-PA and the two hybrid molecules to cells displaying the IL-2 receptor and to cells that do not. Cell killing was studied using a variety of cell lines.
Results: We showed that all of the molecules could be converted to the aerolysin form by proteolytic nicking and that this led to the production of stable oligomers. The R336A variant of proaerolysin did not bind and was only very weakly active against all cell types tested. The IL-2-PA hybrid was active against all cell types, as it could bind to GPI-AP and form functional oligomers. The Il-2-R336A-PA hybrid could not bind to normal GPI-AP positive cells and it had little or no activity against them. Remarkably, this hybrid could bind to cells that display the IL-2 receptor and it was nearly as toxic to these cells as native PA.
Conclusions: The channel-forming protein aerolysin can be targeted to cells displaying the IL-2 receptor. Targeted aerolysin molecules such as IL-2-R336A-PA may have advantages over targeted enzyme toxin molecules in cancer therapy.

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Cancer immunotherapy by Interleukin-21: theoretical evaluation of potential treatment strategies

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Background: The newly-characterized Interleukin-21 (IL-21), a natural derivative of T-helper cells, plays a central role in the transition from innate immunity to adaptive immunity. Murine studies show substantial elimination of various tumors in response to IL-21 application, thereby encouraging its addition into the growing cancer immunotherapeutic arsenal. Still, conditions for efficacious IL-21-therapy, and its conflicting immunostimulatory and immunoinhibitory influence on anticancer cellular responses, are yet to be fully defined.
Methods: We have studied the effects of IL-21 on tumor eradication in a mathematical model focusing on the NK-cell and CD8+ T cell-mediated lysis of tumor cells. To estimate model parameters we used studies in mice inoculated with poorly immunogenic (PI) melanomas, highly immunogenic (HI) fibrosarcomas, or thymoma, and treated with cytokine gene therapy (CGT), hydrodynamics-based gene delivery (HGD), or standard interval dosing (SID) of IL-21. Model accuracy in retrieving tumor growth curves has been validated in independent experiments of melanoma and fibrosarcoma progression in mice treated by IL-21. Putative immunotherapy strategies were simulated and their efficacy was estimated.
Results: Computer simulations accurately retrieved experimental growth dynamics in B16 melanoma, MethA and MCA205 fibrosarcomas, showing a strong dependence of the NK-cell/CD8+ T-cell balance on tumor immunogenicity. Efficient tumor elimination was achieved in melanoma, when simulating an IL-21 dosing regimen that was dynamically-determined according to changes in tumor mass, as in CGT. In contrast, in fibrosarcoma, such a strategy did not prove superior to that of constant dosing protocols, HGD or SID.
Conclusions: Our model analysis supports clinical use of IL-21 as a potent stimulator of cellular immunity against cancer, and suggests selecting the immunotherapy strategy according to tumor immunogenicity. In PI malignancies, but not in HI, IL-21 dosing, at any time, should depend on tumor mass at that time. This method imitates, yet amplifies, the natural anticancer immune response, rather than accelerating only one of the response arms, in an unbalanced manner.

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Targeting brain tumor stem cells with oncolytic virus in combination with temozolomide

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Background: Recently, several groups have described the existence of a cancer stem cell population in human brain tumors. These population is a preferred therapeutic target since has been proposed to be a possible