163 POSTER

Fluorescent imaging of tumor growth, angiogenesis, metastasis and apoptosis using transplantable syngeneic models of murine neuroblastoma

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We have developed a unique transplantable orthotopic model of intraadrenal murine neuroblastoma, as well as models of induced hepatic and/or pulmonary metastasis, and have now incorporated the use of fluorescence-based technologies and differential fluorochrome expression to enable multicolor imaging and/or quantitation of tumor growth, neovascularization, metastasis and apoptosis in these models. TBJ neuroblastoma cells transfected to overexpress the red fluorescent protein (RFP) gene display intense expression in vitro as well as in vivo after orthotopic implantation, or in occult metastatic sites in the liver, lung and/or bone marrow. Gross metastases are more readily visualized in visceral organ sites, and tumors as small as a few cells in size can be imaged, allowing for more sensitive detection of even microscopic residual disease in these organs. TBJ-RFP metastases can be detected not only in visceral organs such as the lung and liver, but also within tumor sections from sites such as the bone marrow. Gross tumor neovascularization can be readily imaged by infusing FITC-dextran (green) in mice bearing red fluorescent TBJ-RFP tumors. To provide a more readily quantifiable model for assessing tumor angiogenesis, we have utilized C57BL/6-TgN (ACTbEGFP)10sb trangenic mice that are engineered to ubiquitously overexpress the green fluorescent protein (GFP). Orthotopic implantation of transplantable UN0092 neuroblastoma cells (newly-derived from C57BL6/J N-myc transgenic mice) into actin-GFP mice leads to the formation of tumors that are vascularized by green fluorescent blood vessels. Concurrent staining with DAPI provides tumor sections in which vascular density (green) can be readily imaged and/or quantitated, and nuclear morphology (blue) can be assessed for apotosis in both endothelial and tumor cell populations. To complement DAPI staining, TBJ cells also have been engineered to constitutively overexpress a fusion construct consisting of GFP linked to BID, a key mediator of mitochondria-dependent pathways of apoptosis. Untreated cells display a diffuse pattern of GFP expression, consistent with cytoplasmic localization of BID, while treatment with agents capable of inducing BID cleavage (i.e doxorubicin), results in subcellular translocation of BID to the mitochondria with a resulting punctate distribution of GFP. We are now using these models to investigate the efficacy and mechanisms of action by new approaches for the treatment of neuroblastoma and have validated the ability of these systems to effectively detect and document specific therapeutic effects including inhibition of tumor growth, neovascularization and metastasis as well as the induction of apoptosis in mice treated with agents including sytemic cytokines and/or small-molecule chemotherapeutics.

164 POSTER

Targeting oncogenic Eph proteins with Hsp90 inhibitors

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Eph receptor tyrosine kinases (RTKs), with 14 distinct members, constitute the largest kinase family. The members of this superfamily regulate cellular boundaries, cell migration, and vascular morphogenesis. Eph RTKs are subdivided into 2 families, based on their sequence homologies and ligand-binding specificities. EphA RTKs bind to ephrin A ligands, while EphB receptors associate with B ephrins. Ligand binding triggers phosphorylation of the C-terminal kinase domain and transmittance of cellular signaling cascades. The Eph RTKs are highly promising targets in a wide variety of cancers, due to their selective overexpression and activation in tumors. EphA2 and several EphB family members have emerged as pivotal players in angiogenesis, invasion, and tumorigenic progression and are correlated with a highly invasive phenotype. Eph RTKs are unique in that the receptors are active in the absence of ligand. Ligand binding has a tumor-inhibitory role for EphA2, resulting in receptor phosphorylation and subsequent protein downregulation mediated by a proteasome-dependent pathway facilitated by the adaptor protein Cbl. In cancer cells, this mode of regulation is lost, as tumorigenic EphA2 receptors do not preferentially associate with ligand, resulting in the overexpression of active receptor. For EphB family members, ligand binding leads to receptor internalization, and, in some cases, activation of tumorigenic pathways. Many oncogenic RTKs interact with heat shock protein 90 (Hsp90), a molecular chaperone involved in mediating the proper conformation and function of a multitude of signaling proteins. We therefore tested whether Eph RTKs were capable of associating with this chaperone. We report that the Eph RTKs examined in this study, EphA2 and EphB1, both associate with Hsp90. This is a significant finding, as Hsp90 inhibitors, such as the clinically-utilized 17-AAG, mediate the proteasome-dependent downregulation of its substrates, or 'client' proteins. Moreover, we demonstrate that the Hsp90-interacting Eph receptors are sensitive to 17-AAG via a degradative pathway that is independent of Cbl. These data demonstrate that Hsp90 plays an essential role in eph RTK function and that pharmacologic agents such as 17-AAG may be exploited as a novel means to downregulate the selective overexpression of these receptors in cancers.

165 POSTER

AZD2171, a potent VEGF receptor tyrosine kinase inhibitor, combined with mechanistically distinct antitumor therapies in vivo

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Background: AZD2171 is a highly potent inhibitor of vascular endothelial growth factor receptor-2 (KDR) tyrosine kinase activity, which is currently in Phase I clinical development. In preclinical studies, once-daily oral dosing of AZD2171 has been shown to inhibit angiogenesis and tumor xenograft growth significantly. The studies described herein, examined the effect of combining AZD2171 treatment with other antitumor therapies *in vivo*.
Methods: AZD2171 (3 mg/kg/day) was combined with the EGFR tyrosine kinase inhibitor gefitinib (Iressa™), the vascular-targeting agent ZD6126, or the topoisomerase I inhibitor CPT-11 (irinotecan) in human tumor xenograft models. The combination of AZD2171 and gefitinib involved co-formulation to enable both compounds to be administered via a single daily oral bolus. When combining AZD2171 (p.o.) with either ZD6126 (i.p.) or CPT-11 (i.v.), AZD2171 was dosed 2 hours prior to administration of the second therapy. Tumor growth inhibition data were examined for statistical significance using a one-tailed *t*-test.
Results: Treatment with either AZD2171 or gefitinib (50 mg/kg/day) inhibited growth of well-established (0.9 cm^3 starting volume) A431 vulval tumor xenografts significantly (95% and 94% inhibition, respectively, after 18 days of dosing; $P<0.001$). When combined concomitantly, these two therapies produced a greater inhibition of tumor growth, with regressions being induced in all tumors (after 18 days of dosing the mean tumor volume was 41% less than the mean pre-treatment volume). In well-established (0.8 cm^3 starting volume) LoVo colorectal tumor xenografts, AZD2171 treatment (day 1–15) restrained tumor growth (62% inhibition at day 15; $P<0.001$) and ZD6126 treatment (100 mg/kg/day, day 1–3) induced a marked growth delay (20% regression was evident at day 7, with subsequent regrowth resulting in 65% growth inhibition at day 15; $P<0.001$). However, a combination of the two induced durable regressions, with the mean tumor volume at day 15 being 60% less than the mean pre-treatment value. AZD2171 (day 1–15) was also examined with CPT-11 (25 mg/kg, day 1 and 8) in LS174T colon tumor xenografts (0.15 cm^3 starting volume). Tumor growth inhibition at day 15 was 35%, 77%, and 98%, for CPT-11 alone, AZD2171 alone, and the combination, respectively ($P<0.001$).
Conclusions: These data indicate that AZD2171 provides added therapeutic benefit when combined with a range of mechanistically distinct therapies in preclinical tumor models.

166 POSTER

A phase I dose-escalation study of anti-a5ß1 integrin monoclonal antibody (M200) in patients with refractory solid tumors

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Background: M200 is an IgG4 chimeric monoclonal antibody directed against a novel integrin target, a5 ß1, on activated endothelial cells. Angiogenesis is initiated within a tumor when one or more of the pro-angiogenic growth factor(s) (e.g., FGF, VEGF, PDGF, etc) is released, resulting in activation of the endothelial cells. These activated endothelial cells then develop new blood vessels by binding to fibronectin in the extracellular matrix via their a5 ß1 receptors. M200 is a potent inhibitor of the a5 ß1 receptor and induces apoptosis of activated endothelial cells *in vitro*, independent of the growth factor stimulus.
Material and Methods: This phase 1 trial was designed to enroll refractory solid tumor patients at escalating doses of 0.5, 1, 2.5, 5, 10, and 15 mg/kg. M200 was infused over 1 hour on days 1, 15, 22, 29, and 36. The endpoints included determining the maximum tolerated dose, dose-limiting toxicity,

safety profile, immunogenicity, pharmacokinetics, and monocyte saturation, as monocytes express the a5 ß1 receptor.
Results: To date, 16 pts have been enrolled and 15 pts have received M200. Baseline demographics for the 15 treated pts were median age=58 years (range 29–81 years), mean ECOG score=1 (range 0–2), and tumor types: colorectal (4), hepatoma (2), melanoma (2), bronchioalveolar carcinoma (1), thyroid (1), parotid (1), renal cell carcinoma (1) breast (1), esophageal (1) and neuroendocrine tumor of the pancreas (1). One pt received 0.5 mg/kg, 2 received 1 mg/kg, 3 received 2.5 mg/kg, 3 received 5 mg/kg, and 6 received 10 mg/kg. No dose-limiting toxicities have been observed. The adverse events that were possibly-related to study drug were mild to moderate nausea (5), fever (2), vomiting (2), headache (2), anorexia (2), and asthenia (2). No pts had an infusion reaction. Two of 16 (13%) pts developed HACA, but there were no apparent associated adverse events. A dose of 10 mg/kg was well-tolerated, achieved monocyte saturation, and a mean trough level of 82 mcg/mL two weeks after the 1st dose, which is above the minimum effective *in vitro* concentration of 2–3 mcg/mL. Thus, 10 mg/kg every 2 weeks is the recommended dosage regimen for subsequent clinical trials. The response outcomes were: SD (9) and PD (6), with 5 of 6 pts who received 10 mg/kg having SD.
Conclusions: M200 appears to be well tolerated at doses up to 10 mg/kg. As dose-limiting toxicity has not been observed, dose escalation is continuing with additional patients to be enrolled into the 15mg/kg cohort. Final data from this study will be presented.

167 POSTER

Combination with PI3 kinase inhibitors allows drastic dose reduction of tumor necrosis factor

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Both in patients treated using the isolated limb perfusion technique and in animal models, Tumor Necrosis Factor (TNF), alone or in combination treatment, displays a very potent antitumor effect. The severe shock-inducing effect resulting from a systemic application of the high doses of TNF needed to obtain the antitumor activity, however, limits its use to locoregional treatment. In mice, we previously could show that the antitumor and shock-inducing activities are not inevitably linked (Cauwels et al., Immunity 13:223, 2000). In order to allow systemic treatment, lowering of the dose of TNF required for tumor destruction is nevertheless necessary. Using transgenic mouse technology, we could show that TNF exerts its antitumor activity by a selective destruction of the tumor vasculature, rather than by a selective cytotoxic effect on transformed cells. This is triggered by an interaction of TNF with the TNF-R1 receptor on endothelial cells. Since PI3kinase (PI3K) is a central component of survival pathways in neovascular endothelial cells, we investigated whether they could be sensitised to the angiodestructive effects of TNF by PI3K inhibitors.
C57BL/6J mice bearing an established tumor (B16, LLC, EL4 or PG19) were treated with daily paralesional injections of murine TNF in combination with wortmannin, or either agent alone, for 10days. In the presence of the latter PI3K inhibitor (0.25 mg/kg, 1h before TNF), the dose of TNF required to obtain complete tumor destruction dropped from 0.35 mg/kg to 0.04 mg/kg. Also human TNF, that, in mice, is a selective TNF-R1 agonist, has a much faster clearance, and can not induce regression of the tumor, turned to be able to cause complete tumor destruction in mice in combination with wortmannin (as was previously shown for the combination of hTNF and IFN-gamma). No synergism was observed when TNF-R1−/− mice were used, while the synergism was retained when tumor cells were used that were rendered insensitive to TNF by a transfection with a dominant negative mutant of TNF-R1. This indicates again that the synergism targets the vasculature rather than the cancer cell in strict sense. Selected key experiments were repeated using the reversible PI3K inhibitor LY 294002, resulting in similar
Conclusions: In groups where higher doses of TNF were used, wortmannin but not LY294002 increased the toxicity of TNF. At lower doses of TNF, effective in the combination treatment, no increase of the toxicity was observed. The PI3K inhibitors used had no or only a marginal growth retarding effect on the tumors, when used alone at the same doses.
Together with some enhancement of the previously established maximal tolerated dose, that could be obtained by supportive measures and/or inhibitors of the toxicity, the order of magnitude of this dose reduction, when also present in human cancer patients, is likely to result in effective doses that are of the same order of magnitude as the tolerated ones. In this respect, it is worthwhile to refer to our recent results, showing that low doses of TNF could enhance the uptake and the effect of doxorubicin encapsulated in long-circulating liposomes (Brouckaert et al., Int. J. Cancer 109:442, 2004), indicating that further combinations with chemotherapeutic agents could result in additional synergisms.

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168 POSTER

PRL-3 promotes invasion in tumor epithelial cells and tube formation in normal endothelial cells

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Phosphatase of Regenerating Liver 3 (PRL-3) has become a drug target in cancer since it was first identified as a marker of colon tumor endothelium (St. Croix et al., Science, 2000) and subsequently recognized as a marker of colon tumor metastasis (Saha et al, Science, 2001; Bardelli et al, Science, 2003). Because of the presence of PRL-3 in two cellular compartments of the tumor, the endothelial and epithelial compartments, future inhibitors of PRL-3 promise a two-pronged attack against tumors. Anti-PRL-3 compounds are expected simultaneously to induce a regression of the tumor vasculature and to interfere directly with epithelial malignancy. We sought to identify the function of PRL-3 in cultured endothelial cells and tumor epithelial cells. We have previously reported that the phorbol ester PMA stimulated PRL-3 mRNA expression and induced proliferation, invasion and tube formation in human microvascular endothelial cells (HMVECs). Given the known upregulation of multiple genes by PMA, it was unclear at the time whether PRL-3 was a major driver of any of these three phenotypes. We now report that HMVECs infected with a PRL-3-expressing adenoviral vector express high levels of the PRL-3 protein and show increased tube formation. These data suggest that PRL-3 was a major driver of the PMA-stimulated tube formation and that it can on its own increase tube formation. These results implicate PRL-3 directly in tumor angiogenesis.
We have previously reported that PRL-3 exogenous expression promoted invasion in the DLD-1 human colorectal adenocarcinoma cell line. We also reported earlier that endogenous PRL-3 mRNA expression correlated positively with invasiveness in the MCF-7 and SKBR3 human breast cancer cell lines and in the SKNAS and IMR-32 human neuroblastoma cell lines in the Matrigel invasion assay. We now report that a PRL-3 siRNA reduces invasion down to 20% of control in the transfected DLD-1 cell model. These data provide additional evidence that PRL-3 stimulates tumor epithelial cell invasion. We also report that the expression of MMP-3 enzyme correlates positively with invasiveness and with PRL-3 protein expression in the transfected DLD-1 cell model as well as in the MCF-7, SKBR3, SKNAS and IMR-32 cell lines, which express PRL-3 endogenously. These data strengthen the evidence indicating that PRL-3 promotes tumor epithelial cell invasion. In addition, these results implicate a possible coregulation of PRL-3 and MMP-3 as a mechanism by which PRL-3 stimulates invasion.
Our studies confirm the involvement of PRL-3 in both tumor angiogenesis and epithelial malignancy. Specifically, our results shed light upon two different functions for PRL-3, depending on which cell type expresses the protein. PRL-3 seems to promote tube formation in endothelial cells, while stimulating invasion in tumor epithelial cells.

169 POSTER

Maximizing the anti-tumor and anti-proliferative effects of 2ME2 by maintaining levels above a threshold concentration for a defined period of time

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2-Methoxyestradiol (2ME2), an endogenous metabolite of estradiol, is an inhibitor of both tumor and endothelial cell proliferation. 2ME2 is currently in oncology clinical trials under the name Panzem®. Various new formulations of Panzem® have been developed recently to increase absorption. These studies were undertaken in an effort to understand the pharmacodynamics of 2ME2, by defining the critical pharmacokinetic parameters associated with inhibition of tumor growth.
MDA-MB-231 human breast carcinoma or Lewis lung carcinoma cell lines were incubated with media alone or increasing concentrations of 2ME2 and inhibition of proliferation was determined by cell counts. Following 48 hr exposure the IC50 values of 2ME2 in MDA-MB-231 and LLC cells are 0.8 μM and 3 μM, respectively. Further studies in which MDA-MB-231 cells were exposed to 2ME2 for limited periods of time each 24 hours demonstrated that a 6 hr incubation with 0.6 μM 2ME2 most closely approached the level of growth inhibition observed following continuous exposure. The level of growth inhibition following exposure of MDA-MB-231 cells to 0.6 μM 2 ME2 for 6 hr could not be mimicked by comparable exposures (AUCs) obtained by altering 2ME2 concentration and incubation time. Moreover, incubation of MDA-MB-231 cells with high concentration