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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.

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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.

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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.

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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,