may play a role in rapamycin-induced apoptosis. Identification of critical molecular markers in tumor cells will help to identify patients who shall benefit from mTOR inhibitors.

Wednesday 29 September

Poster Sessions

New drug targets

36 POSTER

Transcriptional signature associated with sensitivity to ET-743 (Yondelis) in low passage sarcoma cell lines

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ET-743 (trabectedin, Yondelis) is a marine anticancer agent that has shown to induce long lasting objective remissions and tumor control in a subset of patients with pretreated/resistant soft tissue sarcoma. Drug induced tumor control is achievable in a 22% of such patients, but there is not clear indication of the molecular features correlated with clinical sensitivity/resistance to ET-743.

Nine low passage soft tissue sarcoma cell lines explanted from chemo naïve patients with different patterns of sensitivity (IC50 range, 0.4 to 100nM), have been profiled with a cDNA microarray containing 6700 genes relevant in cancer development and drug resistance. The molecular signature of these cell lines was analyzed at baseline and at 4 different time points after ET-743 exposure at the clinically relevant concentration of 10 nM. Additionally, association of p53 mutation and p73 expression levels with ET-743 sensitivity and cell cycle kinetics after treatment were also analyzed.

Gene expression profile analysis revealed upregulation of 86 genes and downregulation of 244 genes in response to ET-743, showing a strong inhibition of gene transcription by the drug. ET-743 gene expression signature reveals a group of genes related with cell cycle control, stress and DNA damage response, such as JunB, ATF3, CS-1. SAT, GADD45B, and ID2 that are upregulated in all the cell lines studied independently of its sensitivity and of the histological subtype.

Transcriptional signature 72 hrs after ET-743 administration, associated with ET-743 sensitivity, showed a more efficient induction of genes implicated in DNA damage response and apoptosis, such as Rad17, BRCA1, PAR-4, p21 and p53DINP1 in the sensitive cell lines group.

Flow cytometry studies showed cell cycle arrest and/or apoptosis in the sensitive cell lines. The presence of p53 mutations correlate with sensitivity. Data produced in this translational program provides with a rational to explore at the clinical level whether this signature can contribute to the identification of the subset of patients that can benefit from ET-743 therapy.

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Sensitivity and resistance of human leukemic blasts to aplidin; molecular signature by gene expression profiling (GEP)

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Aplidin (APLD) is a marine anticancer drug discovered in the Mediterranean tunicate *A. albicans*. The antitumoral activity of APLD has been related to a cascade of events including cell cycle G1 arrest and G2 blockage, an acute apoptotis induction with JNK/p38 sustained activation; and inhibition of VEGF autocrine loop, reducing VEGF secretion and down regulating of the VEGFR-1. The phase I clinical program with APLD has been completed with evidence of a positive therapeutic index and lack of bone marrow toxicity. Phase II clinical studies are currently underway in hematological tumors with special focus on leukemia and multiple myeloma. In vitro and in vivo studies in leukemia models have demonstrated cytotoxicity at concentrations reachable in patients well below the recommended dose, and lack of cross-resistance with conventional agents. A translational program in pediatric acute lymphoblastic (ALL) and acute myeloid (AML) leukemia has produced evidence of variable in vitro sensitivity to APLD in blast from patients (Leukemia 2003, 17: 1338) at concentrations that do no affect normal bone marrow and peripheral blood samples.

Blast cells from 17 ALL and 12 AML patients with differential sensitivity to APLD have been analyzed by Gene Expression Profiling using a cDNA microarray that contains 6700 genes relevant in cancer development, apoptosis and drug resistance. The in vitro sensitivity to APLD of the patient blasts, measured as IC_{75}, ranged from 0.012 to 0.096 mM and 0.011 to 0.153 mM for primary and relapsed ALL, and 0.012 to 0.088 mM for AML, respectively. The IC_{75} median values, used as cut off for classifying the samples as sensitive or resistant were 0.028, 0.014 and 0.045 mM for ALL, ALL-r and AML respectively. Gene expression profiles reveal a specific signature in AML and ALL samples that correlate with the extent of sensitivity to APLD.

AML samples sensitive to APLD presented high expression of genes related to signal transduction, metalloproteases and drug metabolism. Genes in APLD-resistant AML samples are involved mainly in NF-kB activation. In contrast, ALL samples sensitive to APLD presented higher expression of DNA damage response genes.

The GEP model generated in this study will be incorporated in the translational research studies within the phase II program with APLD in resistant leukemia.

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The role of sample preparation on gene expression profiling: impact on clinical use of microarray technology

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Background: Gene expression profiling is quickly entering the clinical arena in areas such as development of new diagnostic markers and pharmacoprediction. Although seminal findings have been reported using transcriptom analysis, numerous challenges remain for its routine usage. One of the foremost is that gene expression patterns in tissues, e.g. tumors or peripheral blood greatly depend on temporal and interindividual variations. In addition, technical aspects of sample processing, isolation of cellular components, RNA preparation methods and other facets such as time from biopsy or blood withdrawal to RNA-isolation and different experimental conditions have been suggested to affect gene expression patterns. However, these issues are poorly investigated in gene expression analysis using microarrays.

Materials and Methods: Peripheral blood from healthy individuals and cancer patients were used as a model to assess the influence of pre-analytical factors on gene expression profiles. Several methods to isolate different cell types and RNA (PAXgene, QIAamp, Ficoll, BD-CPT) and two different blood processing techniques (Buffy Coat vs venipuncture blood) were compared using Affymetrix HG-U133A microarrays. A total of 68 individual array experiments were included in this analysis. Furthermore, the influence of physical factors such as temperature (room temperature, 8ºC), cryopreservation and time delay in sample preparation were also analyzed.

Results: Overall, the pre-analytical conditions have a strong and significant impact on gene signatures outweighing e.g. interindividual differences. Particularly delayed sample handling revealed a striking impact on gene signatures. We observed an induction of genes related to hypoxia, concomitant with down regulation of genes associated with cell cycle, metabolism and apoptosis. Similarly, gene expression was strongly influenced by the choice of cell and RNA preparation technique: e.g. the use of the PAXgene system, solely providing stabilization of the gene expression profile of blood samples, revealed overall decrease of present calls, highest variability and decreased sensitivity for changes in expression patterns of lymphocytes and monocytes. Cryopreservation, different temperatures during cell isolation or the source of the blood sample introduced minor changes, nevertheless, they were biologically relevant as exemplified by regulation of the IL-8 gene by different temperatures during cell isolation.

Conclusions: Clinical utilization of microarray technology will require improved standardization. Careful annotation of sample collection, transportation or storage and of RNA isolation techniques needs to become a prerequisite during clinical use of this technology. Based on our results, we suggest immediate preparation of RNA prior prolonged sample transportation or storage.

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Discovery and development of multiplex angiogenesis inhibitors that target EphB4: validation with a novel chemical-genetics based in vivo model

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EphB4 is a receptor tyrosine kinase (RTK) that plays a critical role in blood vessel development. EphB4 knockout mice die *in utero* from multiple

vascular defects. To determine the role of EphB4 in tumor angiogenesis as well as the therapeutic index for inhibiting EphB4 in the most pharmaceutically relevant setting, we have developed a unique chemical genetics-based mouse model in which wild-type EphB4 is replaced by a functionally intact analog sensitive kinase allele (ASKA) of EphB4 through gene targeting. We have demonstrated that the embryonic lethal EphB4 knockout phenotype is fully rescued in EphB4 ASKA mice, and that ASKA EphB4 is potently and selectively inhibited *in vivo* by the small molecule ASKA inhibitor, 1-NaPP1. These EphB4 ASKA mice are currently being studied in a number of oncology models to determine the effect of specific inhibition of EphB4 on tumor angiogenesis. We have also initiated a drug discovery program to identify small molecule inhibitors against wild-type EphB4. Using medicinal and high-speed analog chemistry, we have created proprietary compound libraries around scaffolds predicted to have kinase inhibitory activity as well as good "drug-like" properties. Screening of these libraries and subsequent medicinal chemistry optimization has generated multiple chemical series of lead inhibitors that demonstrate potent activity in both biochemical and cell-based assays on EphB4. While the compounds show a very favorable selectivity profile (minimal activity on a 25 kinase cross-screen panel), further characterization has demonstrated that they also potently inhibit VEGFR2 and Tie2, two EC RTKs critically involved in tumor angiogenesis. Preliminary *in vivo* testing indicates that these lead compounds have good PK/tox profiles and the ability to inhibit tumor growth. Since tumor angiogenesis is a multi-pathway process, strategies targeting multiple angiogenic kinases are likely to produce maximum clinical efficacy for treating cancer. Multiplex inhibitors of EphB4, VEGFR2, and Tie2, therefore, represent potentially improved new therapies for the treatment of many types of human cancer.

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RAD001 sensitizes tumor cells to cisplatin-induced apoptosis in an mTOR dependent manner by inhibition of p53-induced p21 protein expression

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The use of DNA damaging agents, such as cisplatin, as antitumor agents has revolutionized chemotherapy against a wide variety of solid tumors. However, a narrow therapeutic window combined with the potential for severe side effects has greatly limited their broader application. This has lead to the search for drugs, which sensitize tumors to lower doses of DNA damaging agents, potentially increasing their clinical efficacy. Here we show that RAD001 (everolimus), an orally bio-available derivative of rapamycin currently in phase **II** clinical trials, dramatically enhances cell death when A549 – (lung carcinoma) or MCF7 (breast carcinoma) cells are treated with sub-optimal concentrations of DNA-damaging agents such as cisplatin or gemcitabine. The enhanced loss of cell viability was defined as apoptosis as judged by poly (ADP-ribose) polymerase (PARP) cleavage, a direct measure of caspase 3 activation. Interestingly, wild type status of the tumor suppressor protein p53 (A549/MCF7) correlated with the enhancement of apoptosis since RAD001 was unable to significantly enhance cisplatin-induced cell death in cells lacking (PC3M) or expressing mutant forms of p53 (DU145/HCT15). Through the use of isogenic tumor cell lines generated to stably express either a wild type allele of mTOR or an allele of mTOR that does not bind RAD001, we demonstrate that the effects of RAD001 on both proliferation and the enhancement of apoptosis are directly through the inhibition of mTOR function. Extensive biochemical analysis revealed that RAD001 impeded the induction of the cell cycle regulator p21, a target gene transactivated by p53 as a response to DNA-damage provoked by cisplatin. With the matched tumor cell lines and the use of RNA interference, we further show that the reduced expression of p53-induced p21 is directly responsible for the enhanced sensitivity of the cells to the RAD001/cisplatin combination. Unexpectedly, the effects of RAD001 are not through inhibition of transcription or translation of p21 mRNA, nor through decreased p21 half-life, but instead through inhibition of global translation combined with the high turnover rate of cellular p21 protein. These findings provide the molecular rationale for combining DNA damaging agents with a sensitizing agent such as RAD001, and suggest that such combination strategies will enhance the efficacy of DNA damaging agents in the treatment of cancer patients with solid tumors.

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A potential for combining the rapamycin derivative RAD001 (everolimus) with the EGF/ErbB2/VEGF receptor tyrosine kinase inhibitor AEE788 in human cancer

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RAD001 (everolimus) is an mTOR pathway inhibitor exhibiting potent antiproliferative/antitumor activity, which is currently in phase **II** clinical trials in oncology. AEE788 is a small molecule dual family inhibitor of EGF/ErbB2 and VEGF receptor tyrosine kinases (RTKs) in Phase **I** clinical studies. We addressed the role of the mTOR pathway *in vitro* as a function of ErbB receptor overexpression. The effect of mTOR inhibition on cell cycle progression (characterized by G1 accumulation) was dominant in tumor lines exhibiting low ErbB receptor expression (A549 lung, MCF7 breast). Specifically, exogenous ErbB ligands were unable to bypass the effects of mTOR pathway inhibition despite RTK activation. Strikingly, although two ErbB2-overexpressing lines exhibited a similar phenomenon (BT474 and MDA-MB-453 breast), bypass of the antiproliferative effects of RAD001 was observed in the EGFR/ErbB2- and ErbB2-overexpressing lines MKN7 gastric and SKBR3 breast, respectively. As autocrine receptor activation plays a major role in tumor cell proliferation, these data suggest that the antitumor efficacy of RAD001 could be compromised by the presence of ErbB ligands; arguing for the use of logical drug combination strategies in the context of EGFR/ErbB2-overexpressing tumors. To investigate the potential for RAD001/AEE788 combinations, ErbB2-overexpressing cells (BT474, SKBR3) were incubated with increasing concentrations of AEE788 in the presence of an optimal RAD001 concentration of 2 nM. In both lines, increased antiproliferative effects were observed with the combination as compared to the single agents; with dramatically increased cell death at optimal AEE788/RAD001 concentrations. For example, as assessed by YOPRO analysis following 72 hrs incubation, treatment of SKBR3 cells with 0.8 μM AEE788 (which caused almost total ErbB receptor inhibition) in combination with 2 nM RAD001 resulted in a 29% loss of cell viability (as compared to 0.5%, 4.5% and 0.8% with vehicle-, AEE788- or RAD001-treated cells, respectively). This increased cell death was defined as apoptosis by PARP/Lamin A cleavage analysis, and strongly suggests that RAD001 and AEE788 may elicit more potent antitumor effects in ErbB2-overexpressing tumors when used in combination. Furthermore, a more detailed analysis demonstrated that suboptimal AEE788 concentrations (0.2 μM: which did not totally inhibit ErbB receptor phosphorylation) in combination with 2 nM RAD001, although potentiating G1 accumulation (e.g. G1 population after 24 hrs: 92% combination; 78% RAD001; 66% AEE788; 63% vehicle), had little effect on cell viability as compared to the single agents. Taken together these data indicate that, in order to fully realize the potential of RAD001/AEE788 combinations in cancer patients, it may be necessary to totally inhibit ErbB RTK activity.

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Phase II study of BAY 43-9006 in patients with advanced hepatocellular carcinoma (HCC)

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Background: This multi-centre phase **II** study of BAY 43-9006, a novel Raf kinase and VEGFR inhibitor, was conducted to assess response rate, time to progression (TTP), toxicity, overall survival, pharmacokinetics (PK) and biomarker assessment in patients (pts) with advanced HCC.
Materials and Methods: Pts with inoperable HCC, no prior systemic treatment, Child-Pugh (CP) score A or B, and ECOG performance status =1, received oral BAY 43-9006 at 400 mg bid continuously in 4-week cycles. Tumor response was assessed every two cycles using revised WHO criteria. Biomarker assays (phospho-ERK levels via immunohistochemistry in pretreatment biopsies and Affymetrix gene expression profiling of blood cells from pretreatment draws) were each performed in approximately 25 pts.
Results: Of 137 pts enrolled (M: F=97:40; median age 69 years [range 28–86]), 98 (72%) had CP A and 39 (28%) CP B. Seven (5%) pts had partial