flattened mean steady state plasma concentrations (25−35 μM) across all dose levels. GLI1 was down-modulated >2-fold in surrogate tissue from a majority of pts tested thus far.
Conclusions: The Hh antagonist GDC-0449 was evaluated at 3 dose levels. The unusual pharmacokinetic profile of this oral agent reflects the accumulation of high stable plasma concentrations of GDC-0449. GDC-0449 at 150 mg/day demonstrates clinical efficacy and is tolerated for extended periods of continuous dosing.

356 POSTER

MLN4924, a potent and novel small molecule inhibitor of Nedd8 activating enzyme, induces DNA re-replication and apoptosis in cultured human tumor cells

M. Milhollen[1], U. Narayanan[1], B. Amidon[1], A. Berger[2], S. Langston[3], T. Soucy[4], P. Smith[4]. *[1]Millennium Pharmaceuticals, Molecular and Cellular Oncology, Cambridge, MA, USA; [2]Millennium Pharmaceuticals, Cancer Pharmacology, Cambridge, MA, USA; [3]Millennium Pharmaceuticals, Chemistry, Cambridge, MA, USA; [4]Millennium Pharmaceuticals, Biochemistry, Cambridge, MA, USA*

The initial step in the pathway for conjugation of the ubiquitin-like protein Nedd8 to its cellular targets requires the activity of the Nedd8 Activating Enzyme (NAE). Nedd8 conjugation is required for the proper function of mammalian cullin-dependent ubiquitin ligases (CDLs). These CDLs in turn control the timely ubiquitination and subsequent degradation of many proteins with important roles in cell cycle progression and signal transduction. Inhibition of NAE leads to decreased activity of the CDLs impacting cellular processes relevant to tumor cell growth and survival thereby providing a rationale for targeting NAE as an anti-cancer strategy. MLN4924 is a first-in-class, potent and selective small molecule inhibitor of NAE. This molecule was used to explore the consequences of inhibiting the Nedd8 pathway in cultured human cancer cells.
MLN4924 specifically inhibits Nedd8-cullin formation leading to stabilization of direct CDL substrates by preventing their ubiquitination and degradation through the proteasome. One such CDL substrate is the critical DNA replication licensing factor Cdt1. Over-expression of Cdt1 has been reported to induce DNA re-replication in cells resulting in cell cycle arrest, DNA damage and genomic instability. Immunoflourescence and western blot analysis of HCT-116 cells treated with MLN4924 demonstrated an increase in the nuclear localization and stabilization of Cdt1 preventing its normal cell cycle regulated turnover. Furthermore, cells treated with MLN4924 accumulated in S-phase of the cell cycle with an apparent increase in nuclear size and DNA content coupled with increased BrdU incorporation reflecting over replication of DNA. This aberrant phenotype was associated with the activation of a DNA damage checkpoint response through the ATM/ATR pathway assessed by the expression of elevated levels of phospho-p53 (S15), phospho-Chk1(S317) and phospho-H2AX ultimately leading to cell death through apoptosis. Synchronized cells released into MLN4924 were prevented from progressing into mitosis as demonstrated by a loss of pH3 signal. The continued increase in DNA content >4N and analogous BrdU incorporation associated with the loss of pH3 signal demonstrated that re-replication of the DNA was occurring within the same cell cycle.
The gross accumulation of re-replicated DNA and DNA-damage resulting from re-replication leads to cell cycle arrest and apoptosis demonstrates a novel mechanism of action for MLN4924 in cultured human tumor cells.

357 POSTER

Effects of 5-fluorouracil (FUra)/leucovorin(LV)-induced DNA damage on the Wnt signaling pathway and downstream targets in human colon carcinoma cell lines (cc)

J. Houghton[1], T.S. Jani[1], C. Kundu[1], Y. Jiang[1], J. DeVecchio[1], P. Howe[1]. *[1]Cleveland Clinic Foundation, Cancer Biology, Cleveland, USA*

Sporadic and familial mutations in the APC gene and aberrant Wnt signaling have been demonstrated in the initiation and progression of human colorectal cancer. Activation of Wnt results in the dissociation of the 'B-catenin destruction complex' thereby stabilizing cytosolic B-catenin and facilitating its nuclear translocation. Nuclear B-catenin is subsequently responsible for the transcription of Wnt target genes including cyclin D1 and c-myc. In the absence of Wnt signal or in presence of wtAPC, B-catenin is sequentially phosphorylated by CK1 and GSK3B leading to its ubiquitinylation and subsequent proteosomal degradation. The goal of this study was to determine the effect of FUra/LV treatment on components of the Wnt signaling pathway in the presence of wtAPC or mtAPC and to further evaluate the effect of FUra/LV on survival of these cells.
In a panel of cc, data demonstrated expression of wtAPC in RKO and GC3/c1 and expression of mt APC in HT29. In these cc, FUra/LV caused DNA damage-induced inhibition of clonogenic survival with an IC50 of ~0.5–2.5 uM, reversible by dThd. WtAPC expression was induced in RKO and GC3/c1, while expression of mtAPC was downregulated in HT29 by FUra/LV. Dab-2, a negative regulator of Wnt signaling, demonstrated low expression in RKO, that was upregulated by FUra/LV treatment. Phosphorylation of GSK3B (p-GSK3B) leads to its inactivation, thereby inhibiting B-catenin degradation. Basal levels of p-GSK3B in RKO cells were relatively high and were downregulated by FUra/LV. Further, attenuation in the levels of B-catenin, cyclin D1 and c-myc was also observed, and Wnt signaling was inhibited. These data suggest that FUra/LV elevates the expression of wtAPC and Dab2 and stabilizes the B-catenin destruction complex in RKO cells, thereby causing B-catenin degradation and resultant inhibition of Wnt signaling.
In HT29 cells stably expressing wtAPC in presence of ZnCl2 (HT29-APC), FUra/LV increased the expression of wtAPC and attenuated the levels of B-catenin, cyclin D1 and c-myc. Further, survivin and c-FLIP expression was also reduced and cells were sensitized to FUra/LV-induced apoptosis. No changes were observed in the expression levels of Bcl-xL, cIAP-1 or c-IAP2.
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18F-FLT-PET for the evaluation of MEK inhibitor AZD6244

I. Desar[1], R. Gilles[2], A. Timmer-Bonte[1], M.V. Cantarini[3], C.M.L. van Herpen[1], W.J.G. Oyen[2], W.T.A. van der Graaf[1]. *[1]Radboud University Medical Centre Nijmegen, Department of Medical Oncology, Nijmegen, The Netherlands; [2]Radboud University Medical Centre Nijmegen, Department of Nucleair Medicine, Nijmegen, The Netherlands; [3]AstraZeneca, R&D Alderley Park, Macclesfield, United Kingdom*

Background: AZD6244 (ARRY-142886) is a potent, selective, uncompetitive inhibitor of MEK 1/2 being tested in phase II trials for a number of solid tumours. The RAF/MEK/ERK protein kinase signal cascade is a key intracellular pathway regulating cellular proliferation and survival. A solid oral formulation, incorporating the Hyd-Sulfate salt of AZD6244, is currently being investigated in a phase I study. To assess the effect of AZD6244 on proliferation in tumours, we used the 18F-FLT-PET. 3′-deoxy-3′-[18F] fluorothymidine (18F-FLT), a thymidine analogue PET tracer, which is phosphorylated by the enzyme thymidine kinase (TKI) and is subsequently trapped intracellularly. 18F-FLT trapping is a surrogate marker for proliferation. 18F-FLT-PET was used in this study to investigate if this method can be used as early predictor for patients (pts) on therapy with AZD6244.
Methods: 18F-FLT-PET scans were performed baseline and after two weeks of treatment with AZD6244 Hyd-Sulfate. In this phase I study pts received 25 mg, 50 mg, 75 mg, or 100 mg bid. FLT-uptake in tumours was analyzed qualitatively and quantitatively by measuring SUVmax, SUV50 and SUV70 in regions of interest (ROI). 18F-FLT-PET scans were compared to CT-scans (baseline and after 8 weeks), which were evaluated using the RECIST criteria.
Results: In four pts a baseline and follow up 18F-FLT-PET was performed. One pt with a melanoma showed a qualitative and quantitative decrease of FLT uptake (mean SUVmax baseline 4.6, follow up 2.30). This was followed by a decrease in RECIST of 11% at the CT scan after 8 weeks. Unfortunately, at the second CT evaluation the pt had PD. In one pt with colorectal cancer (CRC) a visual increase of FLT-PET combined with an increase of SUVmax from 1.81 to 4.19 was observed. This pt had SD after the first CT evaluation (RECIST +3.8%) but had PD at the second CT evaluation. The other two pts (one melanoma, one CRC) did not show significant changes in FLT uptake; mean SUVmax baseline 6.46, follow up 5.32 for melanoma pt, mean SUVmax 2.30, follow up 1.37 for CRC patient and had PD (at the first CT evaluation for the melanoma pt or after the second evaluation for the CRC pt).
Conclusion: This is the first report of 18F-FLT-PET to assess the effect of AZD6244, a MEK inhibitor. Changes in SUVmax could be measured. As yet the number of pts is too limited to assess if there is a correlation between the changes in SUVmax and RECIST measurements. Further investigation is warranted.

359 POSTER

A role for tumor associated macrophages in tumorigenesis

A. Bonde[1], S. Zoller[2], R. Schwendener[1]. *[1]Institute of Molecular Cancer Research, University of Zurich, Zürich, Switzerland; [2]Functional Genomics Center Zurich, University of Zurich, Zürich, Switzerland*

Immunocells are known to infiltrate tumor microenvironments and, is the case for most cancers, promote tumorigenesis through various pathways, the best studied being tumor-angiogenesis promoted by tumor associated macrophages (TAMs). However, various intra- and intercellular signaling

pathways in the tumor microenvironment show infiltration dependent alterations in gene expression levels, suggesting infiltrating cells as possible targets for new anticancer therapies. To evaluate this option further we use liposome-encapsulated clodronate (Clodrolip) to specifically deplete macrophages *in vivo*. This tool allows us to study the development of tumors in the presence and absence of TAMs. In this study murine teratocarcinoma tumors (F9 cells) were grown s.c. in Sv129S1 mice. Two groups were studied over the course of three weeks, one control group and one group receiving Clodrolip. Upon immunohistochemical confirmation of macrophage depletion in Clodrolip treated mice, total RNA was extracted from the isolated tumors and subjected to a gene expression analysis. Data obtained from this study confirmed down regulation of macrophage specific markers as well as proinflammatory signaling and proangiogenic factors in TAM-depleted tumors. Moreover, the data indicates TAM-dependent modulations of the extra cellular matrix, cell proliferation, cell adhesion and migratory activity, all of which have been confirmed biochemically. Migration and invasion assays *in vitro* have further confirmed a TAM dependent stimulation of F9 cell aggressiveness. Together these data suggests TAMs to affect basic features of tumorigenesis, supporting the establishment of solid tumors via high tumor cells proliferation activity, highly organized tumor structure and tumor cell migration and extravasation. We are now investigating the molecular mechanisms, through which TAMs may regulate these pro-tumorigenic events. Results obtained from this study will contribute to the understanding and evaluation of TAMs as a possible target for new anti-cancer therapies.

360 POSTER

Ror2 in renal cell carcinoma: evaluating its role in RCC tumorigenesis

T.M. Wright[1], J.D. Gordan[2], W.K. Rathmell[1]. [1]*UNC Lineberger Comprehensive Cancer Center, Curriculum in Genetics and Molecular Biology, Chapel Hill, NC, USA;* [2]*Abramson Family Cancer Research Institute, Department of Cell and Developmental Biology, Philadelphia, USA*

Sporadic renal cell carcinoma (RCC), a notoriously hard to treat solid tumor malignancy, has minimal sensitivity to traditional chemotherapy and immune system modulation. The most current and effective therapies for renal cell carcinoma involve the use of kinase-specific inhibitors targeted against receptor tyrosine kinases (RTK) and ligands specific to angiogenesis signaling. However, though these agents have shown promise, they remain to be fully optimized. Currently, there are no reported cancer cell specific kinases expressed on RCC identified as amenable targets for tumor cell directed therapy. Using a phospho specific RTK screen in renal carcinoma cells, we identified Ror2, an orphan receptor tyrosine kinase previously unknown in renal carcinoma cells. Ror2 is normally expressed in the heart, brain and lungs of developing mice and has also been implicated in the Wnt/β-catenin signaling pathway. Activated kinase expression was observed in RCC cell lines, with Ror2 expressed in a manner dependent on the inactivation of the von Hippel-Lindau (VHL) tumor suppressor and subsequent stabilization of the hypoxia inducible factor (HIF) family of transcription factors. In addition, transcripts of Ror2 were detected in more than 55% of a set of 19 archival human RCC tumor specimens. Among these archival RCC tumor specimens, Ror2 expression also clustered with genes involved in the epithelial to mesenchymal transition (EMT). Additionally, inhibition of Ror2 expression by RNAi not only limited RCC growth in soft agar, a surrogate for invasive cellular growth potential, but also produced fewer tumors in xenografts. Therefore, we hypothesize that we may have identified a kinase involved in key aspects of the transformation process in this malignancy. Further analysis is currently underway to delineate the importance of Ror2 for RCC tumorigenesis and regulation as it represents a potentially important molecular target for RCC.

361 POSTER

FGFR4 Y367C: Contributes to a constitutively active FGFR4 and tumour aggressiveness in breast cancer cell line

P. Foo[1], H.K. Ho[1], S. Streit[1], J.E. Ruhe[1], S. Hart[1], A. Ullrich[1]. [1]*Institute of Medical Biology, Singapore OncoGenome Project, Singapore, Singapore*

Background: FGFR4, member of the fibroblast growth factor receptor family, is an emerging tyrosine kinase cancer target. A polymorphism in the transmembrane domain of the FGFR4 is linked to the progression and worsened prognosis of melanoma, sarcoma and breast-, prostate-, colon- and head and neck carcinoma. Recently, we detected a mutation, FGFR4 Y367C, in the breast cancer cell line MDA-MB-453. Remarkably, a homologous cysteine substitution in FGFR1−3 causes various osteogenic disorders through enhanced dimerization and constitutive kinase activation; and contributes to tumour progression. Hence we set out to determine the role of the FGFR4 Y367C variant in cancer cells and the phenotypic consequences of its inhibition.

Methods: We examined FGFR4 protein expression and activity by western blot analysis and transcript levels by real-time PCR. Full-length FGFR4 was cloned and transfected into Hek293. Cells were treated with cycloheximide (10 μg/mL) for 24 hours, PD173074 (0.1 μM-10 μM) and FGFR4 siRNA for 72 hours.

Results: We found several breast carcinomas highly expressing FGFR4. MDA-MB-453 has a much higher FGFR4 protein expression despite comparable mRNA levels with HuH-7, a cell line with the highest FGFR4 expression among the NCI-60 panel. This high FGFR4 expression is likely due to increased protein stability contributed by Y367C, based on both Hek293 overexpression system and comparing MDA-MB-453 (367C) with HuH-7 (WT). FGFR4 in MDA-MB-453 is also constitutively active. In addition, FGFR4 is the major FGFR isoform in MDA-MB-453, making this cell line an appropriate system to utilise PD173074 (a potent FGFR family inhibitor), as a probe for FGFR4 function in cancer. FGFR4 inhibition with PD173074 and FGFR4 siRNA blocked MDA-MB-453 proliferation. Furthermore, the reduction on downstream MAPK signalling with FGFR4 siRNA indicates the mechanism of FGFR4's proliferative potential in breast cancer.

Conclusion: The novel FGFR4 Y367C variant may confer protein stability that contributes to MDA-MB-453 specific FGFR4 overexpression and highly invasive phenotype. The high expression of FGFR4 in breast carcinoma and anti-proliferative effect of PD173074 and FGFR4 siRNA provide compelling evidence to consider FGFR4 as a therapeutic target against breast cancer.

362 POSTER

STAT3 protein binding to supercoiled DNA

S. Chasovskikh[1], O. Timofeeva[1], A. Dritschilo[1]. [1]*Lombardi Cancer Center, Radiation Medicine, Washington, DC, USA*

Background: Signal transducer and activator of transcription 3 (STAT3) proteins modulate various physiological functions including cell-cycle regulation, apoptosis and cell survival by regulating gene expression. Upregulation of phospho-STAT3 activity or increases of unphosphorylated STAT3 levels have been observed in cancers. Both forms of STAT3 are able to regulate gene transcription and contribute to tumor progression. The phosphorylated STAT3 forms a dimer and binds to IFNγ-activated sequences (GAS) on DNA. It is not clear yet whether unphosphorylated STAT3 binds to its own DNA site or regulates gene expression through interaction with other transcription factors. Atomic Force Microscopy (AFM) allows visualization of structural relationships in the interaction between protein and DNA. In this study we used AFM to analyze binding of unphosphorylated STAT3 to DNA to better understand the mechanism of STAT3-dependent transcriptional regulation. In addition, we studied a truncated STAT3 isoform, generated by proteolytic processing at the C-terminus, to elucidate whether distinct transcriptional activities of the different STAT3 isoforms are due to differences in DNA binding.

Materials and Methods: Recombinant STAT3 protein was mixed with supercoiled GAS-containing plasmid and topoisomers of plasmid DNA (pUC8F14) in 50 mM Tris-HCl, 10 mM NaCl buffer with pH 8.0. Freshly cleaved mica was incubated in a mixture of a 1-(3-aminopropyl)silatrane (APS) solution for 30 min to prepare APS-mica. The protein-DNA complexes were deposited on APS-mica for 2 min, then washed with deionized water, and dried with nitrogen gas. The AFM images were obtained using the NanoScope IIIa instrument equipped with an E-scanner (Digital Instruments, Santa Barbara, CA) and analyzed by the accompanying software in the imaging module.

Results:

1. Binding to GAS full sized STAT3 dimer
2. Binding to hairpin full sized STAT3, and 67.5 kDa

We observed that the 67.5 kDa STAT3 fragment binds to the ends of the hairpin arms of the cruciform structure on DNA containing 4 unpaired nucleotides. Full length STAT3 proteins bind to the four-way junction region in the cruciform structure. Statistical analyses of the volume distributions of STAT3 molecules in DNA-STAT3 complexes are interpreted to show that the 67.5 kDa STAT3 fragments form predominantly monomers or dimers with cruciform structures. The STAT3 proteins form predominantly dimers or tetramers with the cruciform structures of the plasmid DNA.

Conclusion: Secondary DNA structures, such as cruciforms, can create new protein binding sites and potentially block the movement of the transcription-elongation complex. The affinities of the truncated forms and full length unphosphorylated forms of STAT3 to secondary structures of DNA provide additional insight into mechanisms that may underly STAT3 protein effect on gene regulation. Accordingly, inhibition of STAT3 DNA binding may offer a novel therapeutic target for gene regulation.