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**Phase I assessment of mechanistic pharmacodynamic biomarkers for MLN8054, a small-molecule inhibitor of Aurora A kinase**

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**Background:** The inhibition of Aurora A kinase causes defects in centrosome separation, spindle assembly and chromosome alignment, leading to transient mitotic delays in dividing cells. Despite these mitotic delays, cells lacking functional Aurora A undergo division, with deleterious consequences such as cell-cycle arrest and death. The molecular sequelae of Aurora A inhibition has been used to develop indirect mechanistic biomarkers. This study examines the application of these biomarkers to the assessment of Phase I pharmacodynamics of MLN8054, a small-molecule inhibitor of Aurora A.
**Materials and Methods:** Patients (pts) with advanced solid tumors were enrolled at centers in Spain and the United States. MLN8054 was administered orally in cycles of 7 to 21 days (25−80 mg/day QID) followed by 14 days of rest to cohorts of 3−6 pts. Skin biopsies (2−3 mm) and tumor biopsies were obtained before day 1 dosing and 7 days later. Skin and tumor biopsies were evaluated histopathologically using H&E staining, and assessed for mitotic counts (an indirect readout of Aurora A inhibition) by staining for the mitotic marker pSer10 histone H3. Tumor biopsies were further evaluated for morphological readouts of Aurora A inhibition by examining chromosome alignment and spindle bipolarity. Multi-focal plane images of tubulin- and DNA stained samples were acquired using an automated fluorescence microscope and 3D reconstructions of mitotic cells were generated. Multiple scorers were presented with blinded individual mitotic spindles in a random order to assess chromosome alignment and spindle biopolarity, and the majority call was assigned to each mitotic spindle.
**Results:** Evaluable skin biopsies pre- and post-treatment were available from 63 pts. Of these, 44 demonstrated an increase in mitotic counts after dosing relative to baseline, a change consistent with Aurora A inhibition. Evaluable tumor biopsies pre- and post-treatment were available from 10 pts. The majority of these demonstrated reduction in chromosome alignment and in spindle bipolarity after dosing relative to baseline; consistent with Aurora A inhibition. In this dataset, there was a high degree of concordance between all of the assays used, and between the result of the skin and tumor assays.
**Conclusions:** Taken together, these data provide compelling indirect evidence of the inhibition of Aurora A by MLN8054 in patient tumor and skin tissue.

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**Expression of leptin receptor is an independent prognostic marker of Middle Eastern colorectal carcinomas**

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**Background:** The adipokine leptin and its receptor (Ob-R) are expressed in various cancer tissues including colorectal carcinomas (CRC). High levels of leptin are seen in obesity that is a risk factor for colon cancer. Incidence of colorectal carcinomas is increasing in Saudi Arabia and a high prevalence of obesity has been reported.
**Materials and Methods:** We investigated the role of Ob-R and its relationship with PI3K/AKT activation in a large series of CRC tissues in a tissue micro array (TMA) format followed by in vitro studies using a panel of CRC cell lines. Immunohistochemical of Ob-R and various PI3-kinase/AKT pathway proteins on 448 CRC samples in TMA setting. MTT and flow cytometeric assays were performed to access cell proliferation and apoptosis.
**Results:** Ob-R over expression was associated with an early AJCC stage ($p = 0.0305$), well differentiated tumors ($p = 0.0001$), pM0 tumors ($p = 0.0396$) and histology subtype of adenocarcinomas ($p = 0.0009$). CRC with Ob-R expression showed significantly better overall survival than those with the low Ob-R expression ($p = 0.0005$) remained an independent prognostic factor in multivariate analysis. Our in vitro studies showed that Leptin increases proliferation of CRC through Ob-R that mediates the PI3-kinase/AKT signaling pathways. Leptin treatment of CRC cell lines induces activation of AKT and FOXO1 and expression of Ob-R specific siRNA prevented leptin-induced activation of AKT and its down-stream targets.
**Conclusions:** Leptin plays a critical role in CRC growth and survival through PI3K/AKT pathway via Ob-R. Ob-R expression is an independent prognostic marker and might represent a novel therapeutic target for CRC treatment.

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**A comparative analysis of megakaryocyte potentiating factor and mesothelin as serum markers for the detection of malignant pleural mesothelioma**

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**Background:** An early and reliable blood test is one deficiency in diagnosis of malignant pleural mesothelioma (MPM). Megakaryocyte potentiating factor (MPF) and mesothelin variants (MSLN), members of the mesothelin gene family, have been studied as candidate serum markers for MPM. We developed a novel enzyme-linked immunosorbent assay (ELISA) system to compare the diagnostic efficacy of MPF and MSLN as serum markers for the specific detection of MPM.
**Material and Methods:** Serum samples were collected from 27 consecutive patients with non-resectable MPM. The patient population included 13 with epithelial type MPM, three with sarcomatoid type, five with mixed type and six with unclassified type (diagnosed by cytological analysis). For controls, we used 47 patients with lung cancer, 35 with other cancers (18 ovarian, 8 stomach and 9 colon cancers), 9 asbestos-exposed asymptomatic subjects and 38 healthy adults without a history of asbestos exposure. The serum concentrations of MPF and of the soluble form of mesothelin were measured by each specific sandwich ELISA.
**Results:** Statistically significant elevation of serum MPF and MSLN levels was detected in MPM patients in comparison with every control group. The area under the receiver operating characteristic curve (AUC) was calculated for differentiation of MPM, lung cancer, healthy asbestos-exposed subjects and healthy adults. While the AUC for serum MPF was 0.879, cutoff = 19.1 ng/ml (sensitivity = 74.1%, specificity = 90.4%), the AUC for serum MSLN was 0.713, cut-off = 93.5 ng/ml (sensitivity = 59.3%, specificity = 86.2%). A comparison between AUC for MPF and MSLN values showed that MPF is significantly superior to MSLN ($p = 0.025$).
**Conclusions:** Our study shows that MPF is a significantly more sensitive and specific serum marker for the detection of malignant pleural mesothelioma compared with MSLN.

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**Potential importance of the ceramide pathway in the action of the tumour vascular disrupting agent ASA404 (DMXAA, 5,6-dimethylxanthenone-4-acetic acid)**

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**Background:** The tumour vasculature is regarded as an important target for cancer chemotherapy and tumour vascular disrupting agents (tumour VDAs) are receiving increasing attention as components of combination chemotherapy protocols. ASA404 is a tumour VDA with a dual mode of action involving both direct effects on the tumour vasculature and indirect effects mediated by induction of cytokines. ASA404 has completed Phase II evaluation and, on the basis of promising activity, has commenced a Phase III trial in combination with carboplatin and paclitaxel for the treatment of non-small cell lung cancer. The availability of appropriate biomarkers to detect the action of tumour VDAs is of great relevance and we have previously demonstrated both in mice and in cancer patients that administration of ASA404 increases in plasma concentrations of serotonin (5-HT) and its hepatic metabolite 5-hydroxyindole acetic acid (5-HIAA). 5-HT is a platelet product and it would be advantageous to identify products of tumour tissue that increase in response to ASA404. Tumour cells, macrophages and vascular endothelial cells all respond to cytokines and other signalling molecules by hydrolysis of membrane sphingolipids to ceramides, which in turn are converted to sphingosine and released from cells. We investigated the hypothesis that treatment of mice with ASA404 led to increases in plasma sphingosine.
**Methods and Materials:** Liquid chromatography (LC) methods were previously developed for 5-HIAA and LC-triple quadrupole mass spectrophotometric methods were developed and validated for sphingosine. C57Bl mice, with or without Colon 38 carcinomas, were treated with ASA404 and plasma samples collected for analysis.
**Results:** Treatment with ASA404 at a therapeutic dose (25 mg/kg) resulted in a time-dependent increase in plasma sphingosine concentration, which was much larger in tumour-bearing mice than in non-tumour bearing

mice. Treatment of mice with sphingosine (25 mg/kg) resulted in a time-dependent rise in 5-HIAA.
**Conclusions:** The results, together with those from work in progress, are consistent with the hypothesis that ASA404 activates the ceramide pathway. Ceramides and/or sphingosine may activate downstream targets including p38 MAP kinase, leading to vascular damage and consequent release of 5-HT from platelets. In addition to its role in ASA404 action, sphingosine may be a potentially useful biomarker for the assessment of patients treated with ASA404 in combination therapy.

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### Analysis of EGFR and KRAS mutations in circulating tumor DNA (cTDNA) from plasma of NSCLC patients in phase 2 trials of XL647

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**Background:** EGFR and KRAS mutations play a critical role in the response to EGFR tyrosine kinase inhibitors (TKI) in NSCLC. Obtaining adequate tumor tissue in patients (pts) with non-operable disease, especially prior to initiation of a later line of therapy when the molecular profile of a cancer may have evolved, is a key element for the development of targeted anticancer agents and remains challenging. Sensitive assay methodology, eg allele specific PCR, to analyze cTDNA may facilitate development of targeted agents such as XL647, a TKI which potently inhibits EGFR (including the T790M resistance mutation), HER2 and VEGFR2 in vitro.
**Methods:** EGFR and KRAS mutations were determined in plasma cTDNA by the sensitive Scorpions ARMS technology (DxS Ltd, Manchester, UK). Samples were collected from pts enrolled in NSCLC clinical trials of XL647 either in a first line or late line of therapy after previous EGFR TKI benefit. EGFR and KRAS mutations in plasma were compared to those in available tumor samples.
**Results:** Plasma samples (n = 67) from 55 pts were analyzed in the DxS EGFR29 panel. A subset of 46 samples was analyzed with the DxS KRAS kit. EGFR ($p < 0.0001$) and KRAS ($p = 0.0003$) DNA levels were lower in plasma from late line pts compared to those with newly diagnosed disease. The spectrum of EGFR mutations detected included Exon19 deletions, L858R, T790M, L861Q, and G719X. Assay failure was more likely when testing KRAS (19/46) than EGFR (3/67), $p < 0.0001$ and more common when testing KRAS in the late line setting (16/23 vs 3/23), $p < 0.0001$, thought secondary to lower PCR performance specific to the KRAS sequence. When testing an individual patient over time, variability was noted. The significance of this observation is being explored. Data from 11 paired plasma and tumor samples in first line pts showed concordance for EGFR status at 82%, specificity at 100%, while the sensitivity to detect EGFR mutations was limited at 50%. In the late line, EGFR mutations were detected in plasma from 11/39 pts. Correlation of these results relative to direct analysis of archival tumor is ongoing.
**Conclusions:** cTDNA can be informative for EGFR and KRAS mutation testing; however, the current technology performs better on samples from newly diagnosed patients than on those with disease recurrence/persistence. Optimal timing for plasma sampling and larger studies are required to better understand the limitations of mutational analysis conducted exclusively in plasma.

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### DUSP4 expression level in colorectal primaries predicts overall survival benefit in Kras wild-type and Kras mutant colorectal cancer after treatment with cetuximab for metastatic disease

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**Background:** DUSP4 dephosphorylates and inactivates ERK and is upregulated by Kras mutations (MUT). Higher expression of DUSP4 was found in epithelial cell cultures with activated ras (Bild et al. Nature 2005). Microarray analysis on metastases of colorectal cancers (CRC) treated with cetuximab (CTX) (Khambata-Ford S et al. JCO 2007) identified DUSP4 as a top resistance marker. We correlated Kras MUT state and DUSP4 mRNA expression level in 197 primary CRC of patients (pts) treated with CTX +/− irinotecan (IRI) with objective response (OR), progression-free survival (PFS) and overall survival (OS).
**Material and Methods:** Tumor areas were manually dissected on FFPE samples. Kras codon 12&13 MUT were analyzed by an allelic discrimination assay. We measured DUSP4 mRNA expression by real-time qRT-PCR. Relative expression levels were calculated comparing average values of duplicate reactions with a calibrator and normalizing them to 3 housekeeping genes: GAPDH, RPL13, UBC. Levels were expressed as calibrated normalized relative quantities. We used the median of these values as cut-off to distinguish high from low expression.
**Results:** 45% had a Kras MUT. Kras wild type (WT) was associated with OR ($p < 0.0001$), PFS ($p = 0.0005$) and OS ($p = 0.0001$). Low DUSP4 was associated with Kras WT ($p < 0.0001$). DUSP4 was associated with OR: 72% (33/46) of responders had a low DUSP4 compared to 44% (66/151) of non-responders ($p < 0.0012$). Overall, there was no association between DUSP4 and PFS ($p = 0.33$). In Kras WT, there was a trend towards longer median PFS in low DUSP4 ($p = 0.10$). Overall, OS was associated with low DUSP4 [41w (95%CI [36−49]) vs 33w high DUSP4 (95%CI [27−46]) ($p = 0.06$)]. In Kras WT, OS was associated with low DUSP4 [54w (95%CI [430−72]) vs 34.5w high DUSP4 (95%CI [25−54]) ($p = 0.01$)]. In Kras MUT, OS was associated with high DUSP4 [32w (95%CI [15−33]) vs 18w low DUSP4 (95%CI [21−39]) ($p = 0.02$)]. A Cox regression model for PFS and OS was built using Kras, DUSP4 and skin toxicity (table).

| | PFS | | | | OS | | | |
|---|---|---|---|---|---|---|---|---|
| | Sig. | HR | 95% CI | | Sig. | HR | 95% CI | |
| | | | Lower | Upper | | | Lower | Upper |
| Kras WT | | | | | | | | |
| DUSP4 (< or > median) | 0.0308 | 0.614 | 0.395 | 0.956 | 0.0024 | 0.507 | 0.327 | 0.786 |
| Skin tox (gr 2−3 vs 0−1) | <0.0001 | 0.345 | 0.223 | 0.533 | <0.0001 | 0.407 | 0.265 | 0.623 |
| Kras MUT | | | | | | | | |
| DUSP4 (< or > median) | 0.1030 | 1.514 | 0.920 | 2.492 | 0.0772 | 1.582 | 0.951 | 2.631 |
| Skin tox (gr 2−3 vs 0−1) | 0.1576 | 0.678 | 0.395 | 1.162 | 0.0060 | 0.443 | 0.248 | 0.792 |

**Conclusions:** DUSP4 expression levels influence OS in both Kras WT and Kras MUT CRC treated with CTX +/− IRI. In Kras WT low DUSP4 levels are favourable, while in Kras MUT high DUSP4 levels are. Kras WT with high DUSP4 may have other oncogenic MUT e.g. Kras codon 61. The extent of ERK signalling is shown not to be the same in all Kras MUT. The prognostic effect of DUSP4 levels needs to be looked into, but the use of DUSP4 expression level as a substrate for the extent of ERK signalling may make it possible to select CRC likely to benefit from ERK-inhibitors.

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### Models for response to the MEK inhibitor GSK1120212 confirm RAS and BRAF mutations as predictive biomarkers and suggest other, unexpected tumor types for clinical evaluation

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The growth factor stimulated RAS/RAF/MEK/ERK signaling pathway is involved in many aspects of cancer progression, and is activated in a large percentage of tumors evidenced by frequently occurring RAF and RAS mutations in cancer. BRAF mutations occur in ~45% of melanomas and KRAS in ~90% of pancreatic adenocarcinomas. GSK1120212 is a potent and selective allosteric inhibitor of the MEK1/2 enzymes that exhibits antiproliferative effect in cell lines and xenograft models. To identify predictive biomarkers to facilitate the clinical development of this compound, sensitivity profiling was carried out for over 300 cancer cell lines. The cell lines were from diverse tumor types to confirm existing hypotheses and explore the sensitivity of other tumor types that were not initially considered to be sensitive to MEK inhibition. As expected, BRAF/RAS mutation status emerged as a strong predictor of response. Cell lines from tumor types that have high occurrence of BRAF/RAS mutations (e.g., melanoma, pancreas and colon cancer) showed a higher rate of sensitivity. We further refined the predictors by grouping cell lines into cytotoxic and cytostatic groups. GSK1120212 has a cytostatic effect in cell lines that are RAF/RAS mutant with co-occurring PI3K/PTEN mutations but cytotoxic in the absence of PI3K/PTEN mutations, suggesting mutant PI3K/PTEN are cytotoxicity resistance markers for GSK1120212. However, for certain tumor types that do not carry BRAF or RAS mutations, sensitivity