Comprehensive Predictive Biomarker Analysis for MEK Inhibitor

GSK1120212

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Abstract
The MEK1 and MEK2 inhibitor GSK1120212 is currently in Phase II/III clinical development. To identify predictive biomarkers, sensitivity to GSK1120212 was profiled for 218 solid tumor cell lines and 81 haematological malignancy cell lines. For solid tumors, RAF/RAS mutation was a strong predictor of sensitivity. Among RAF/RAS mutant lines, co-occurring PIK3CA/PTEN mutations conferred a cytostatic response instead of a cytotoxic response for colon cancer cells which have the biggest representation of the co-mutations. Among KRAS mutant cell lines transcriptomics analysis showed that cell lines with an expression pattern suggestive of epithelial-to-mesenchymal transition were less sensitive to GSK1120212. In addition, a proportion of cell lines from certain tissue types not known to carry frequent RAF/RAS mutations also appeared to be sensitive to GSK1120212. Among these were breast cancer cell lines, with triple negative breast cancer cell lines being more sensitive than cell lines from other breast cancer subtypes. We identified a single gene DUSP6, whose expression was associated with sensitivity to GSK1120212 and lack of expression associated with resistance irrelevant of RAF/RAS status. Among haematological cell lines, AML and CML cell lines were particularly sensitive. Overall, this comprehensive predictive biomarker analysis identified additional efficacy biomarkers for GSK1120212 in RAF/RAS mutant solid tumors, and expanded the indication for GSK1120212 to patients who could benefit from this therapy despite the RAF/RAS wild-type status of their tumors.

Introduction
The RAS-RAF-MEK-ERK (MAPK) signalling pathway is one of the most activated and also most extensively studied pathways in cancer (1). Genetic alterations causing aberrant activation of members of the MAPK pathway are commonly observed in cancer. For instance, receptor tyrosine kinases such as EGFR, HER2 or c-MET are frequently activated as a result of DNA amplification or gain-of-function mutations(2-4). Activating mutations in RAS family genes, most often in KRAS, are found in about 30% of cancers (1). BRAF is mutated in more than 40% of melanomas, and is also mutated at lower frequencies in other tumor types such as colon cancer and ovarian cancer. Therefore, while mutations in MEK1 or MEK2 are rare, pathway activation caused by mutations in the RTKs, RAS or RAF are mediated through MEK kinases, making inhibition of MEK an attractive cancer therapeutic strategy (1).

Targeted therapies rely on the phenomenon of oncogene addiction to attempt to inactivate a mutated oncogenic pathway, critical to survival of cancer cells while sparing normal cells which do not carry the mutation and are not similarly addicted to the pathway (5, 6). Thus predictive markers identifying the appropriate genetic background are crucial to the success of targeted therapies. Examples include translocations of BCR-ABL with imatinib, and ERBB2 DNA amplification with trastuzumab and lapatinib (7-9). Various reports suggest that activating mutations in the RAS or BRAF genes represent the most important tumor predictive biomarkers for sensitivity to MEK inhibitors (10, 11). However, there is clear variance in sensitivity even among the limited set of RAF/RAS mutant cell lines that have been profiled. Further, early clinical data indicates that although RAF/RAS mutant tumors (e.g. melanoma) appear to be more sensitive, tumors with these mutations are not uniformly responsive (12, 13). In some cases,
co-occuring genetic alterations presumably provide mechanisms of resistance. A more comprehensive profile of the genetic determinants of sensitivity and resistance may further increase response rates to MEK inhibitors, and provide rational combination strategies. Furthermore, it may assist in identifying those tumors that are dependent on MAPK pathway activation but do not harbor a RAF/RAS mutation. Identifying RAF/RAS wild-type tumors that might be responsive to MEK inhibitor would enable more cancer patients to benefit from these inhibitors.

GSK1120212 is a potent and specific allosteric inhibitor of MEK1/2 that is currently in phase II/III clinical development (14). It has shown clinical activity in tumors with activating mutations in BRAF (ASCO 2010). In this study, we conducted a comprehensive predictive biomarker study for GSK1120212 using 218 solid tumor cell lines covering different tissue types and subtypes. A comprehensive genetic characterization has been performed in these cell lines. Our data confirm the association of RAF/RAS mutations with sensitivity to GSK1120212 treatment. Additionally, we observe that among RAF/RAS mutant cell lines, co-occurring PIK3CA and PTEN mutations appear to promote a cytostatic rather than a cytotoxic response. Further, among KRAS mutant, PIK3CA/PTEN wild type lines, those with expression markers characteristic of having undergone transition to a more mesenchymal phenotype tend to be less sensitive. Among RAF/RAS wild-type breast cancer lines, we observed that basal breast cancer cell lines tend to be more sensitive than luminal cell lines. For the majority of the tumor types with or without RAF/RAS mutations, the expression of DUSP6 gene is associated with sensitivity and lack of the expression is associated with resistance. Sensitivity to GSK1120212 was also profiled for 81 hematological malignancy cell lines, and we observed that a majority of AML and CML cell lines were sensitive to GSK1120212. This paper provides a comprehensive analysis of the markers contributing to both sensitivity and resistance to GSK1120212, a selective MEK1/2 inhibitor.

**Materials and methods**

**Cancer cell lines**

Cell lines were purchased from the American Type Culture Collection [ATCC] and the German Resource Centre for Biological Material [DSMZ]. The majority of the cell lines were used within 6 months of acquisition and no re-authentication was performed. For the DSMZ cell bank STR DNA typing is performed for authentication and numerous authentication tests are performed at the ATCC cell bank (STR, Sequencing, SNP fingerprinting). DNA copy number profiling and transcriptomics profiling on these cell lines were described previously (15). Briefly, unless otherwise recommended, cell lines were cultured in RPMI-1640 supplemented with a final concentration of 10% fetal bovine serum, 2 mM GlutaMAX™ and 1 mM sodium pyruvate. Genomic DNA was extracted from each line using Mini DNeasy kit (Qiagen, Inc., Valencia, CA, USA) and copy number data was profiled using Affymatrix 500K SNP Chip. RNA was isolated from exponentially growing cells by replacing the media with trizol and purifying the RNA using Qiagen RNeasy spin columns. The transcriptomics data from these cell lines were collected using the Affymetrix U133 Plus2 Gene Chips in triplicate. All the genomic data on these cell lines have been deposited in [https://cabig.nci.nih.gov/caArray_GSKdata/](https://cabig.nci.nih.gov/caArray_GSKdata/). Mutation data of these cell lines for
BRAF, KRAS, NRAS, HRAS, PIK3CA and PTEN were mostly obtained from COSMIC database (v49 release, Wellcome Trust Sanger Institute, United Kingdom).

**Cell line proliferation assay**

Each cell line was seeded into 384-well microtiter plates at high and low cell densities, ranging from 300 to 3600 cells per well, depending on cell line doubling rate. Cells were plated in triplicate and incubated at 37°C in 5% CO2 for 24 hours. Compound was added at 10 concentrations, ranging from 0.16 nM to 5 µM along with a DMSO control. Cell numbers were measured using DAPI nuclei staining and quantitated with an InCell1000 High Content Analyzer (GE Biosciences, Little Chalfont, UK). A zero-time value (D₀) was measured for each cell line, at each seeding density, immediately after the addition of DMSO control. Cell numbers at day 3 (D₃) were also measured using DAPI staining. A drug response curve was generated using model 205 of XLfit in Microsoft excel. GI50 was defined as the drug concentration at which cell growth was inhibited at 50% compared to DMSO control. Compound cytotoxic effect was reached if the number of cells after 3 day drug treatment (D₃) is smaller than that measured at D₀ (i.e. cell number at D₃< cell number at D₀) and compound cytostatic effect was reached if the number of cells did not decrease below the number measured at D₀ following up to 5 µM drug treatment. For haematological lines, a 3-day proliferation assays was conducted using CellTiter-Glo (Promega).

**Western analysis**

After media was aspirated from the dishes, cells were rinsed once with cold PBS and were then scraped off plates in cold PBS and spun down. Cell pellets were lysed in M-PER lysis buffer (Thermo Scientific, Rockford, IL) supplemented with Protease inhibitors (Roche Diagnostics, Mannheim, Germany) and phosphotase inhibitors (Upstate). Cell lysates boiled in loading buffer were run on 4-12% gels (NuPage), transferred to PVDF membrane, and probed with antibodies. The antibodies utilized in the studies are: anti-phospho-ERK at 1:1000 dilution (Cell signaling, cat.# 4376); anti-phospho-AKT at 1:1000 dilution (Cell signaling, cat.# 9271); anti-cleaved Parp at 1:1000 dilution (Cell signaling, cat.# 9541); anti-total ERK at 1:500 dilution (Santa Cruz, cat.# sc-93); anti-total AKT at 1:1000 dilution (Cell signaling, cat.# 2967); anti-GAPDH at 1:5000 dilution (Sigma-aldrich, cat# G8795); anti-DUSP6 at 1:500 dilution (Santa Cruz, cat# sc-8599)

**Transcriptomics analysis for epithelial/mesenchymal features and breast cancer cell line characterization**

Based on Affymetrix U133_2 transcriptomics data from the cell lines (described previously) transcript abundance was estimated by normalizing all probe signal intensities to a value of 150 using the Mas5 algorithm in the Affymetrix Microarray Analysis Suite 5.0. For subsequent analysis, the average probe intensity was used for sample triplicates. For determining epithelial or mesenchymal profile, 4 epithelial markers (CDH1, OCLN, DSP, CLDN1) and 6 mesenchymal markers (S100A4, CDH11, VIM, CDH2, ACTA2, FN1) were selected for analysis. The affymetrix probes assaying each were interrogated. The probe with the highest expression values across the cell lines was selected where more than one probe existed for a gene and the probes were further checked for specificity by BLASTing against the human genome. The selected probes identifiers were 201131_s_at, 227492_at, 200606_at, 222549_at, 2031869_s_at,
207173_x_at, 201426_s_at, 203440_at, 200974_at, 212464_at. The intensity values (averaged across the triplicates) were Z-score normalised across all the cell lines. An average of the epithelial markers and mesenchymal markers were taken. If the epithelial average was higher than the mesenchymal average the cell line was classified as epithelial and if the mesenchymal average was higher, the cell line was classified as mesenchymal.

For breast cancer cell lines, basal and luminal cell lines were identified based on hierarchical clustering. Basal breast cancer cell lines were differentiated from luminal cell lines based on previously published signatures (16).

Results

Sensitivity profiling of MEK1/2 inhibitor, GSK1120212, in solid cancer cell lines

To identify predictive biomarkers for GSK1120212, 218 solid cancer cell lines encompassing different tissue of origins were profiled for sensitivity using a 3-day proliferation assay (Supplementary Table S1). Cell lines were defined as sensitive if gI50 was below 50nM and resistant if above 1μM while if between 50nM and 1μM the sensitivity of the cell lines was considered intermediate. As demonstrated for other MEK inhibitors, GSK1120212 showed increased activity against RAS or RAF mutant cell lines compared to cell lines wild-type for these genes (Fig. 1A). When segregated into tissue types, tumor types known to carry high frequency of RAF/RAS mutations, such as pancreatic cancer, colon cancer and melanomas, showed generally increased sensitivity to MEK inhibitor, GSK1120212 (Fig. 1B).

However for certain tissue types known to carry low frequency of RAF/RAS mutations, a significant proportion of cell lines showed sensitivity to GSK1120212. Among these were breast, glioma, head and neck, kidney and ovarian cancer. The biological determinants of the sensitivity of these RAF/RAF wild-type cell lines were further analyzed and discussed in subsequent sections.

MEK1/2 inhibitor caused a cytostatic rather than cytotoxic response on RAF/RAS mutant tumors encoding co-occurring PI3K or PTEN mutations

Based on gI50, more than 80% of RAF/RAS mutant lines would be considered sensitive to MEK1/2 inhibitor GSK1120212 (gI50<50nM). However, since drug sensitivity in preclinical studies commonly overpredicts the observed clinical response (17), we attempted to further refine the sensitivity determinant for tumors harboring RAS or RAF mutations. To that end an additional criteria to gI50 was added where cell line response to GSK112212 was categorized as cell killing (cytotoxic= cell number at D3<D0) or cell growth inhibition (cytostatic= cell number at D3>D0). Since mutation in the PI3K pathway was reported to be associated with resistance to MEK inhibitors (18), PIK3CA and PTEN mutation status was collected for all cell lines tested (Supplementary Table S1). Among RAF/RAS mutant cell lines, only colon cancer cell lines were significantly represented carrying co-occurring PIK3CA/PTEN and RAF/RAS mutations. For the colon cell lines with concomitant RAF/RAS and PIK3CA or PTEN mutations, the response to GSK1120212 was primarily cytostatic (9 out of 10) instead of cytotoxic (1 out of 10).

Accordingly, 4 out of 5 RAF/RAS mutant colon cancer cell lines with gI50<50nM and responded in a cytotoxic manner to GSK1120212, were wild-type for PIK3CA/PTEN (Fig. 2A). Taken together, these data suggest that in a RAF/RAS mutant background, PIK3CA or PTEN mutations are predictive of cytostatic rather than cytotoxic response to GSK1120212.
To evaluate whether cell lines that harbour concomitant mutations in the PI3K and MAPK pathways depend on both activated pathways, we conducted studies with GSK1120212 and a PI3K inhibitor GSK1059615 (19), or a combination of the two drugs. Treatment of a colon cell line RKO (BRAF and PI3K mutant) with GSK1120212 or GSK1059615 alone significantly reduced p-ERK or p-AKT levels respectively, indicative of pathway inhibition. However neither compound alone caused substantial apoptotic cell death as evidenced by non-detectable cleaved PARP (c-PARP). In contrast, when cells were treated with both compounds (200nM GSK1120212 and 5μM of GSK615), p-ERK and p-AKT were reduced to non-detectable levels, and c-PARP was readily detected, suggesting cell death (Fig. 2B). These results suggest that the presence of concomitant activating mutations can permit alternative redundant growth or survival signals that may reduce sensitivity to a therapy targeting a single pathway, while co-treatment with inhibitors of each pathway cause cell death.

**KRAS mutant lines with a mesenchymal expression pattern are less sensitive to GSK1120212**

Analysis of GSK1120212 sensitivity of cell lines with MAPK pathway mutations demonstrated that while most BRAF mutant cell lines respond with single digit nM gI50s, KRAS mutant cell lines exhibited a wider range of sensitivity (Fig. 3A). In order to further define the determinants of KRAS mutant cell line sensitivity to GSK1120212, additional genetic and transcriptomic analysis were performed. At the genetic level, we observed a trend for KRAS mutant cells with co-occurring PIK3CA or PTEN mutations to be less sensitive to GSK1120212 (Fig. 3A). However this is not statistical significant (t-test). Epithelial to mesenchymal transition (EMT) has been implicated in resistance to multiple cancer therapies including EGFR inhibitors (20). Furthermore, Singh et al. (21) showed that KRAS mutant cell lines sensitive to RNAi mediated KRAS depletion had higher levels of e-cadherin protein, an epithelial state marker, than KRAS mutant lines insensitive to KRAS RNAi depletion. The process of EMT was studied using transcriptomics analysis for the KRAS mutant cell lines. A ten gene transcript signature designed to assay epithelial and mesenchymal status was derived and used to correlate with response of KRAS mutant cell lines to GSK1120212. Using this gene transcript signature we demonstrated that cell lines with increased mesenchymal features showed higher gI50s (less sensitive) than KRAS mutant cell lines with increased epithelial features (t-test p=0.00162) (Fig. 3B). The gene expression levels from which the EMT prediction was derived are represented in the heatmap at bottom of Figure 3B. Exclusion of PIK3CA/PTEN mutant cell lines from this analysis improved the significance of this association (p=0.00902) (Fig. 3C), suggesting that epithelial like (by transcriptional analysis) KRAS mutant cell lines wild-type for PIK3CA/PTEN are more sensitive to GSK1120212. Interestingly, despite the high sensitivity of most BRAF mutant lines regardless of PIK3CA/PTEN mutation or mesenchymal-like features (Supplementary Table S3) (Fig. 3D), three BRAF(V600E) mutant sarcoma cell lines (GCT, A673 and SW-982) were not sensitive. Sarcomas typically arise from transformed mesenchymal cells, and the three cell lines have a mesenchymal-like gene signature.

**Basal or triple negative breast cancer cell lines showed increased sensitivity to GSK1120212 compared to other breast cancer subtypes**
While breast cancer cell lines carry low frequency of RAF/RAS mutations a significant number were sensitive to GSK1120212 (Fig. 1B). To better understand the determinants of breast cancer cell line sensitivity to GSK1120212 we correlated the HER2, ER and PR status as well as basal or luminal status defined by published transcriptomics signature (16) to sensitivity to GSK1120212, as demonstrated in Figure 4. A total of 9/12 (75%) of more sensitive cell lines were of the basal subtype, while only 2/9 (22%) resistant lines were basal ($p = 0.029$). Notably, 3/4 (75%) of HER2 amplified cell lines were less sensitive, and 9/11 (82%) of triple negative (HER2, ER and PR negative) cell lines were more sensitive, suggesting the ‘triple negative’ subtype may preferentially respond to GSK1120212. Although basal breast cancers are not equivalent to triple negative breast cancers, they largely overlap in the clinic (22).

**DUSP6 expression is a predictive biomarker of MEK inhibitor sensitivity**

While multiple gene signatures have been derived to predict MAPK pathway activation or response to MEK inhibitor (10, 23, 24), the technical difficulty to clinically measure these multiple transcripts has hindered their utility and usage. Our search to narrow the number of transcripts to predict cellular response to GSK1120212 led to identification of DUSP6 as single transcriptional marker of MEK inhibitor sensitivity. Of special interest is the fact that presence or absence of DUSP6 expression appears to be associated with MEK inhibitor sensitivity, not just degree of expression. DUSP6 is a cytoplasmic phosphatase inactivating pERK2 and is transcriptionally upregulated following activation of the MAPK pathway (25). Transcriptomics profiling of DUSP6 expression revealed that its expression was associated with sensitivity to GSK1120212 (Sensitivity: 79%; specificity: 82%; $p$-value =0.0027) (Fig. 5A). Notably, among head and neck (H&N) cancer cell lines, none of the five sensitive cell lines carry RAF/RAS mutations. However, they all express high levels of DUSP6 (Supplementary Table S1 and Supplementary Table S2), and the only H&N cancer cell line not expressing DUSP6 was resistant to GSK1120212. Moreover, among the 7 tested cervical cancer cell lines the 4 cell lines that do not express DUSP6 were all resistant to GSK1120212, and the remaining 3 cell lines expressing DUSP6 showed an intermediate response to GSK1120212. Among ovarian cancer cell lines, RAF/RAS mutation status alone would correctly predict 3 out of 4 sensitive cell lines. A2780 had a sensitive response to GSK1120212 with $\text{gI50}$ of 28nM, and although it is wild type for RAF/RAS, it has a high expression of DUSP6. None of the 3 resistant ovarian cell lines express DUSP6 (MAS5 signal below background of 100) (Supplementary Table S2) (Fig. 5B). Western blot against DUSP6 was performed on cell extracts from 7 ovarian cancer cell lines and demonstrated that levels of DUSP6 protein correlated with that of DUSP6 mRNA levels and sensitivity to MEK GSK1120212 inhibitor (Fig. 5C). In addition, treatment of a sensitive cell line OVCAR5 with GSK1120212 led to dose and time dependant protein expression decrease of DUSP6 (Fig. 5D), suggesting that DUSP6 has the potential to be simultaneously a predictive (high endogenous level) and pharmacodynamic (decreased expression upon drug treatment) marker of GSK1120212 sensitivity.

**Among hematopoietic malignancies cancer cell lines from AML and CML showed increased sensitivity to GSK1120212**

GSK1120212 was profiled against 81 cancer cell lines from haematological malignacies (Fig. 6). As with the solid tumor lines, activating mutations in RAF/RAS were predictors of sensitivity to GSK1120212, with 12 of 22 sensitive cell lines encoding mutations in NRAS, KRAS, HRAS or BRAF (Supplementary Table...
Interestingly, majority of AML lines tested showed sensitivity to GSK1120212 (11/12 cell lines), which can only be partly explained by RAF/RAS activating mutations (6/12 lines with RAF/RAS mutation). Similarly, the majority (4/6) of CML lines tested were sensitive to GSK1120212 but none encoded activating mutations in RAF/RAS. In both cases, sensitivity to GSK1120212 in the absence of RAF/RAS mutations may be explained by other commonly occurring oncogenic events (BCR-Abl translocation in CML, and FLT3 internal tandem duplications in AML) that have been previously shown to signal through the MAPK pathway (26, 27). While AML and CML lines tested were predominantly sensitive to GSK1120212, the majority of B-Cell lymphoma lines (Burkitt, Hodgkin’s, NHL, and various other subtypes), ALL (B-Cell or B-cell precursor), multiple myeloma, T-cell derived ALL, and CTL cell lines were resistant to GSK1120212. Somewhat surprisingly, although 4/7 T-cell derived ALL lines had activating mutations in KRAS or NRAS, only one line was sensitive to GSK1120212. Taken together, these data demonstrated that AML and CML cancer cell lines are more sensitive to GSK1120212 than other hematopoietic cancer cell lines, in which sensitivity correlates weakly with RAF/RAS mutational status.

Discussion

Clinical response has been seen with GSK1120212 in BRAF and RAS mutant tumors (ASCO 2010). However, only a subset of BRAF and RAS mutant tumours responded to the MEK inhibitor GSK1120212 (ASCO 2010). These results are reminiscent of the clinical response to Herceptin, where only a subpopulation of HER2 amplified breast cancers show benefit with treatment (28). Based on these data, it is imperative that additional biomarkers to BRAF/RAS mutation be identified to improve the clinical success rate and benefit to patients. On the other hand it is also important that we discover predictive markers that can identify sensitive patients with wild type RAF/RAS tumors, broadening the therapeutic benefit of MEK inhibitors.

With these goals in mind, we performed a comprehensive predictive biomarker analysis using response data for GSK1120212 from approximately 300 cancer cell lines. As with previously characterized MEK inhibitors (10, 11), we observed that activating RAF/RAS mutations were the predominant predictors of sensitivity. Consequently, tumor types known to carry high frequency of RAF/RAS mutations (melanoma, pancreatic and colon cancers), thus particularly depend on MEK pathway activation for growth, showed the highest rate of response (Fig. 1B). Among RAF/RAS mutant colon cancer cell lines, cell lines encoding both RAF/RAS and PI3K/PTEN mutations tend to have a cytostatic (inhibition of cell growth) rather than a cytotoxic (cell death) response (Fig. 2A). This observation was further confirmed with RKO colon cancer cell line (BRAF/PI3K dual mutant) demonstrating significant cell death only following treatment with both MEK and a PI3K inhibitors but with neither agent alone (Fig. 2B).

AKT/PI3K and MAPK are the two most important cancer pathways downstream of most tyrosine kinase receptors. Crosstalk between these two pathways has been reported (29) and it is conceivable that for cell lines with mutations in both pathways, activated AKT can confer survival signal upon MEK pathway inhibition while simultaneous inhibition of both pathways causes cancer cell death. This observation not only suggests combination studies using a PI3K inhibitor and a MEK inhibitor, but also carries significance in further stratifying colon cancer patients for response. Approximately 40% of colon cancer
patients’ tumors have BRAF or RAS mutations, and half of these tumors would have concomitant PI3K/PTEN mutations, while the other half would be wild-type for PI3K/PTEN and would have greater probability to respond to single agent MEK inhibitor (30, 31).

Mutant KRAS can activate multiple downstream effector pathways (29) besides the MEK pathway. As a result, the consequence of inhibiting MEK pathway in RAS mutant tumors can be influenced by the activity of other pathways downstream of RAS. In contrast, MEK pathway seems to be the only physiologically relevant pathway activated in BRAF mutants (32). Therefore, cells with mutant BRAF would presumably be truly ‘addicted’ to MEK pathway activation. In an earlier study using CI-1040, it was reported that only BRAF mutation predicted sensitivity to MEK inhibition but not RAS mutation (11). Using GSK1120212, we found that overall cell lines with RAS mutations tend to be more sensitive (Fig. 1A). However among 27 BRAF V600E mutant cell lines (the non-V600E mutations are known to have less activity), most were highly sensitive with GI50 of 5nM or below (20/27, 74%) regardless of PI3K/PTEN status. On the contrary, only 13/40 (32.5%) KRAS mutant cell lines gave GI50s of 5nM or below, and a wider range of response was observed (Supplementary Table S3).

Since there is potential for some but not all patients with RAS mutant tumors to benefit from MEK inhibitor therapy, it is important to identify predictive biomarkers in the RAS mutant background. Even with GI50 alone, there appeared to be an association between PI3K/PTEN mutation and higher GI50, although this is not statistically significant (Fig. 3A). A previous study using RNAi to knockdown KRAS in KRAS mutant cell lines showed that not all KRAS mutant cell lines require KRAS to maintain cell viability (21). The gene signature derived from this study to predict ‘true’ KRAS dependency was associated with epithelial phenotype. Through analysing mRNA expression patterns we found that KRAS mutant tumors with an expression pattern suggesting more mesenchymal features tended to be more resistant (Fig. 3B and 3C). This relationship may be mirrored in the observation that the only BRAF V600E mutant cell lines that showed even moderate resistance were derived from sarcomas, a mostly mesenchymal tumor type. In a recent publication on predictive biomarkers for another MEK inhibitor AZD6244, the authors observed an association between Wnt pathway and resistance in KRAS mutant colon cell lines (24). Both MAPK and Wnt pathway activation are known to play an important and interconnected role in EMT transition in cancer maybe through RKIP (33) or BMP4. However, despite this observed relationship between epithelial or mesenchymal expression pattern and MEK sensitivity, the underlying mechanism is not clear. EMT transition has been shown to be an acquired resistance marker to chemotherapy (20) and EGFR inhibitors (34) in multiple epithelial cancers (20). It could be that more advanced tumors tend to have undergone EMT and also to have gathered more upregulated growth pathways which may compensate for MEK inhibition. Another possible explanation is that the MAPK pathway is an epithelial lineage pathway and perhaps as cells progress through EMT and become more mesenchymal they also become less dependent on the MAPK pathway. This would suggest that RAF/RAS mutation would not be a route to oncogenesis in haematological malignancies. RAF/RAS mutations are less common but certainly not absent from this class of cancers.

We decided to profile cell lines derived from many different tissue types and found tumor types that are known to have a low frequency of RAF/RAS mutations but unexpectedly showed sensitivity toward
Predictive biomarkers identifying tumors without mutations in RAF/RAS gene that still respond to MEK inhibition offer the opportunity to allow more patients to benefit from GSK1120212. All the available mutation data for the RAF/RAS wild-type cell lines was collected from Sanger database in hope of finding some other mutations that can explain the observed sensitivity. However no statistically significant association was observed, probably because most of the mutations occur at low frequency. Among breast cancer subtypes, triple negative breast cancer is considered to be EGFR driven and requires MEK pathway activation (35). Indeed, triple negative breast cell lines showed higher sensitivity toward GSK1120212 comparing to breast cell lines from other subtypes (Fig. 4). For other tumor types, we investigated expression of MEK pathway genes downstream of RAF/RAS to identify tumors that have an activated MEK pathway but not identifiable with a RAF/RAS mutation. Multiple transcriptomics signatures have been reported either for RAF/RAS mutations or sensitivity toward MEK inhibitors (10, 23, 24). Most of these signatures include a DUSP gene, a family of dual-specificity phosphatases that regulate the MEK pathway. We found that a single gene DUSP6, a DUSP that specifically dephosphorylates and inactivates ERK2, predicts response to GSK1120212 with sensitivity of 79% and specificity of 82% (p-value= 0.0027) (Fig.5). DUSP6 is known to be involved in a feedback loop with ERK2 at the transcriptional level via ETS2 (25). Presumably its expression would reflect ERK2 activity and thus MAPK pathway activation status. In fact it is among the genes identified by Pratilas et al to be the transcriptional output of MEK pathway (36). Indeed, when the MAPK pathway was inhibited by treatment with GSK1120212, a sharp reduction in DUSP6 level was also observed (Fig. 5D). Measuring a single gene instead of a group of genes in the clinical samples requires less tumor material. The fact that in cell lines DUSP6 transcription expression is usually below detection (in resistant cells) or significantly expressed (in sensitive cells) makes the assay almost binary. This has a significant advantage translating to clinical specimens when cellular heterogeneity of tumors complicates the interpretation of multi-factorial gene signatures. In cell lines, the protein level of DUSP6 correlated well with mRNA level (Fig. 5C), making it a possibility to use immunohistochemistry to measure DUSP6 levels. With the sharp reduction of DUSP6 expression upon GSK1120212 treatment, it also has the potential to be used as a pharmacodynamic marker, as well as a predictive marker.

Among haematological malignancies, the growth of the majority of myelogenous lines (AML and CML) was inhibited at low concentrations of GSK1120212 with gI50s of <50nM. These sensitive lines included RAF/RAS mutant and wild-type AML, while all of the CML lines were RAF/RAS wild-type. Among these tumors, other frequent oncogenic events including BCR-Abl translocation and FLT3 internal tandem duplications can lead to alternative routes to constitutive RAS activation and MAPK pathway dependency (26, 27).

Overall, we have conducted a comprehensive, in-depth predictive biomarker study for the MEK inhibitor GSK1120212 in solid tumor cell lines as well as cell lines from haematological malignancies. This work has identified additional predictive biomarkers in cancer cell lines with a RAF/RAS mutant background as well as biomarkers that would identify RAF/RAS WT tumors that might respond to GSK1120212. The ultimate validation of these data will be the analysis of these biomarkers in cancer patients who have received GSK1120212.
References


Figure legends
Figure 1. The response to GSK1120212 was profiled in a panel of 218 solid cancer cell lines using a 3-day proliferation assay. The cell lines were defined as sensitive if GI50 was below 50nM, and resistant if GI50 was above 1μM. GI50s between 50nM and 1μM were considered having an intermediate response. **A)** Cell line response to GSK1120212 in relation to BRAF or RAS (i.e. KRAS, NRAS and HRAS) mutation status; **B)** Cell line response to GSK1120212 in relation to different tissues of origin.

Figure 2. RAF/RAS mutant colon cancer cell lines with co-occurring PIK3CA/PTEN mutations gave more frequent cytostatic instead of cytotoxic responses. **A)** For the 18 RAF/RAS mutant colon cell lines, cytotoxic response was defined as cell number at D3<D0 and cytostatic response was defined as cell number at D3>D0. Cell lines were grouped based on PIK3CA and PTEN mutation status, with cell lines encoding PIK3CA or PTEN mutations labelled as ‘Mut’, and cell lines wild type for both genes as ‘WT’. For the colon cell lines with concomitant RAF/RAS and PIK3CA or PTEN mutations, the response to GSK1120212 was primarily cytostatic (9 out of 10) instead of cytotoxic (1 out of 10). **B)** A colon cancer cell line RKO (BRAF and PIK3CA mutant) was treated with either GSK1120212 (GSK212) at 200nM or a PI3K inhibitor GSK1059615 (GSK615) at 1μM or 5μM as single agents or in combination. After 30 hours of drug treatment, cell lysates were collected and westerns were performed for p-ERK for MAPK pathway activation, p-AKT for PI3K pathway activation, or c-PARP for cell death. Treatment of cells with GSK1120212 or GSK1059615 alone significantly reduced p-ERK or p-AKT levels respectively, with total proteins unchanged. However substantial apoptotic cell death was only observed when cells were treated with both compounds.

Figure 3. GI50s for GSK1120212 are represented on the y-axis and individual cell lines on the x-axis. **A)** KRAS mutant, PIK3CA/PTEN mutant lines are colored red, showing a slight tendency for these lines to have higher GI50s than KRAS mutant, PIK3CA/PTEN wild type lines (not statistically significant, t-test). **B)** KRAS mutant lines are coloured for their epithelial/mesenchymal transcriptional signature, lines colored green have higher average expression of the epithelial marker genes CLDN1, DSP, OCLN and CDH1, than the mesenchymal marker genes, FN1, ACTA2, CSH2, VIM, CDH11 and S100A4. Lines colored orange have higher average expression of the mesenchymal marker genes compared to the epithelial marker genes. Lines with a mesenchymal signature tend to have higher GI50s (p=0.00162). Below the bar chart is a heatmap showing the Z-score normalised expression values of the markers genes in a heatmap. Higher expression is denoted by green, lower expression by red and the median by blue. **C)** KRAS mutant, PIK3CA/PTEN wild type lines are colored for their epithelial/mesenchymal transcriptional signature. Lines with a mesenchymal signature tend to have higher GI50s (p=0.000902). **D)** BRAF mutant lines are colored for their epithelial/mesenchymal transcriptional signature. Sensitive lines have both epithelial and mesenchymal signatures. The 3 most resistant lines have mesenchymal signatures.
Figure 4. Proliferation response, measured by GI50, for GSK1120212 in 21 breast cancer cell lines indicates that basal breast cancer cells are more responsive than luminal cell lines (depicted in blue and red bars respectively). A total of 9/12 (75%) of more sensitive cell lines were of the basal subtype, while only 2/9 (22%) resistant lines were basal (p = 0.029). ERBB2 amplification, ESR positivity and PR positivity (by RNA levels) are denoted for each cell line. Notably, 3/4 (75%) of ERBB2 amplified cell lines were less sensitive, and 9/11 (82%) of triple negative (ERBB2, ESR and PR negative) cell lines were more sensitive.

Figure 5. DUSP6 expression was associated with sensitivity to MEK inhibitor GSK1120212. A) The 218 solid cancer cell lines with GSK1120212 sensitivity data were grouped based on GI50 response, with ‘S’ being sensitive (GI50<50nM); ‘I’ being intermediate (GI50 between 50nM and 1μM) and ‘R’ being resistant (GI50>1μM). DUSP6 mRNA expression was based on cell line transcriptomics data, with MAS5 signal above background value of 100 as being expressed (filled bar) and MAS5 signal below 100 as being lack of expression (empty bar). DUSP6 mRNA expression was significantly associated with sensitivity toward GSK1120212 (p-value = 0.00266169). B) The 11 ovarian cancer cell lines with GSK1120212 response data were grouped based on GI50 response. All the sensitive ovarian cancer cell lines expressed DUSP6 mRNA, and none of the resistant ovarian cancer cell lines expressed DUSP6 mRNA. C) The protein lysates from 4 sensitive and 3 resistant ovarian cancer cell lines were tested for DUSP6 protein expression by western. Only the 4 sensitive ovarian cancer cell lines showed detectable levels of DUSP6 protein. The DUSP6 mRNA expression represented as MAS5 signal from transcriptomics data, as well as the sensitivity to GSK1120212 were also labelled for each cell line. D) A sensitive ovarian cancer cell line OVCAR5 was treated with 40nM or 400nM of GSK1120212, and protein lysates were collected after different time points. Treatment with GSK1120212 led to dose and time dependent protein expression decrease of DUSP6.

Figure 6. Growth inhibitory effect of GSK1120212 on various haematological malignancy cell lines. A set of 81 cell lines from haematological malignancies were treated with GSK1120212 in a 3-day growth assay. While the majority of cell lines were insensitive (GI50>1μM) or immediately sensitive (GI50: 50-1000nM), the majority of AML and CML lines showed GI50 below 20nM. AML: acute myeloid leukemia; ALL: acute lymphocytic leukemia (B-Cell & B-Cell precursor); BCL: B-Cell Lymphoma; NHL: Non-Hodgkin’s Lymphoma; DLCL: diffuse large-cell lymphoma; various: lymphomas include follicular, mantle cell, Pleural-effusion, diffuse small-cell lymphoma; Burkitt: Burkitt’s lymphoma; CML: chronic myeloid leukemia; HL: Hodgkin’s lymphoma; MM: multiple myeloma; T-ALL: T-cell acute lymphocytic leukemia; CTL: cutaneous T-Cell leukemia.
Figure 1

A.

B.
Cytotoxic

Cytostatic

ncer cell lines
(RAF/RAS mutant)

\[ \text{Mut} \quad \text{WT} \]

A.

PIK3CA/PTEN

B.

Figure 2

\( \text{t-AKT} \quad \text{t-ERK} \quad \text{C-PARP} \quad \text{P-AKT} \quad \text{P-ERK} \)

GSK212
GSK615

- +
- +
- +
- +

# colon cancer cell lines

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Figure 3
Figure 5
Figure 6

GI50 (nM in log scale)
Molecular Cancer Therapeutics

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