410 POSTER

Insulin stimulation of head/neck squamous cancer cells confers resistance to the anti-proliferative effects of an EGFR kinase inhibitor

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Although most squamous cell cancers of head/neck (HNSCC) overexpress epidermal growth factor receptors (EGFRs), these tumors respond infrequently to treatment with EGFR kinase inhibitors (EGFR-TKI). In other tumor types, resistance to EGFR-TKIs has been associated with increased expression of IGF-1 receptors. These receptors may generate EGFR-independent growth factor signals that limit the anti-tumor effects of EGFR-TKIs. To test this hypothesis in HNSCC cells, we studied the SCC-25 cell line. Treatment of serum-deprived cells with the EGFR-TKI PD158780 produced a dose-dependent inhibition of growth (IC50=300nM). This correlated with a decrease in the proportion of cells in S-phase at 24 hours from 0.21 to 0.11, as measured by flow cytometry. Immunoblots with phospho-specific antibodies showed that PD158780 treatment blocked constitutive low-level phosphorylation of the EGFR and the potential downstream effectors Gab1, Shc, and Erk. However, there was little effect on the level of spontaneously phosphorylated Stat3. Thus, EGFR-independent signals constitutively activate stat3 despite the fact that treatment of the cells with exogenous EGF increased phosphorylation of stat3 as well as the other proteins. The SCC-25 cells also expressed moderate levels of IGF-1 receptor protein. We attempted to activate the receptors with high concentrations of insulin rather than IGF-1 since these cells are reported to produce high levels of IGF-1 binding proteins. Insulin (0.1; 1uM) treatment produced a modest increase in cell proliferation (1.5X after 48 hours) but also abrogated the negative effects of PD158780 on cell growth and on cell cycle progression. In contrast, EGF (0.1;1nM), IGF (1;10nM), or VEGF (5;50 ng/ml) had little or no influence on drug sensitivity in these assays. Short-term stimulation with insulin (5 min) substantially increased phosphorylation of AKT1 but had minimal effects on Erk or Stat3 phosphorylation, either in the absence or presence of PD158780. Taken together, these studies show that signaling by the IGF-1 and/or insulin receptors in HNSCC cells can abrogate the growth suppressive effects of EGFR kinase inhibition. The mechanism remains to be determined but the ability of insulin to selectively increase AKT phosphorylation suggests that in SCC-25 cells, activation of the upstream phosphatidyl-3-inositol kinase (PI3K) pathway plays a role. Also, constitutive phosphorylation of stat3 may be required but is not sufficient to confer resistance to EGFR-TKIs.

411 POSTER

Pharmacodynamic evaluation of the mTOR inhibitor AP23573 in phase 1 clinical trials

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Background: AP23573 is a novel non-prodrug analog of rapamycin that inhibits mTOR signaling in tumors, which leads to cell cycle arrest, tumor cell shrinkage and inhibition of angiogenesis. mTOR inhibition results in a decrease in the phosphorylation and activity of a number of critical signaling proteins including 4EBP1. We have developed a pharmacodynamic (PD) marker of AP23573 action in peripheral blood mononuclear cells (PBMCs) that is being utilized in ongoing phase 1 trials in cancer patients (pts).
Materials and Methods: Two phase 1 dose escalation trials are being conducted in pts with advanced malignancies to evaluate the safety, tolerability and maximum tolerated dose of AP23573 administered IV using 2 dosing regimens (weekly, and daily × 5 every 2 weeks [QDx5]). The trials also are designed to evaluate potential PD markers of AP23573 activity in PBMCs and to estimate AP23573 pharmacokinetic (PK) parameters. Whole blood samples are being collected for PD analysis at a subset of time points designated for PK sampling. For PD analysis, protein extracts were prepared from PBMCs and analyzed by Western blot using antibodies specific for 4EBP1 phosphorylated at Ser65/Thr70 (P-4EBP1).
Results: A robust P-4EBP1 signal was reproducibly detected in extracts from PBMCs obtained from volunteers, and ex vivo incubation with AP23573 led to a dose-dependent decrease in phosphorylation with a 50% reduction in signal at ~3 ng/mL. PD analysis has been carried out on samples from 8 patients dosed on the weekly schedule (6.25–100 mg) and 10 patients dosed on the QDx5 schedule (3–28 mg). In all patients, P-4EBP1 levels were reduced by at least 90% within 1 h after infusion of AP23573. In pts dosed on the weekly schedule, P-4EBP1 levels remained reduced by >70% 48 h after dosing, with this level of inhibition persisting in some pts for 7 days. Similarly, in pts dosed on the QDx5 schedule, P-4EBP1 levels remained reduced by >70% 72 h after the last dose, with this level of inhibition persisting in some pts for 10 days. AP23573 levels >10 ng/mL generally correlate with >70% inhibition of P-4EBP1.
Conclusions: We have developed a sensitive PD assay that demonstrates rapid and prolonged P-4EBP1 inhibition in PBMCs of pts administered AP23573. Ongoing analysis of additional pts will help determine the relationship between the dose of AP23573 and the duration of the PD response. This information will be used to guide dosing regimens for future trials.

412 POSTER

A phase I and pharmacokinetic study of AEE788, a novel multi-targeted inhibitor of ErbB and VEGF receptor family tyrosine kinases

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Background: An open label phase I dose escalation study of AEE788 is ongoing which evaluates safety, preliminary efficacy, pharmacokinetics (PK) and pharmacodynamics in patients (pts) with advanced cancer. AEE788 is an orally active, reversible, small molecule multi-targeted kinase inhibitor with potent inhibitory activity against ErbB and VEGF receptor family of tyrosine kinases. It is extensively metabolized in the liver by cytochrome P-450 3A4 and forms significant amounts of an active metabolite, AQM674, in humans. AQM674 has *in vitro* pharmacologic activity similar to parent AEE788. 27 pts with advanced cancer received daily doses (qd) of AEE788 at 25, 50, 100, 150, or 225 mg. No DLT has been observed. Mild skin rash and diarrhea have been reported at ⩾ 100 mg/day.
Methods: A 24-hour PK profile was obtained on days 1, 15 and 28, with trough sampling on days 8 and 22 to determine drug serum concentrations (conc) using a validated LC/MS/MS assay. The PK parameters of AEE788 and AQM674 were computed by non-compartmental methods.
Results: Serum conc of AEE788 and AQM674 were highly variable; coefficients of variation were on average 70% in Cmax and AUC. Serum conc of parent and metabolite increase as dose and dose duration increase. AEE788 exposure increases overproportionately with dose. Cmax was reached 2–5 and 3–7 hours post dose for AEE788 and AQM674, respectively. The metabolite serum conc profile appears to reflect relative changes in parent, suggesting rapid metabolite formation and elimination (⩾ parent). The mean metabolite/parent (M/P) ratio is 0.7 (range 0.2 to 2), and appears to decline with dose and/or dose duration. In patients where the accumulation index could be measured, the mean among dose groups was 3.5 (range 2–6) for parent and 3 (range 2–5) for metabolite. The effective half life, estimated from the accumulation index, exceeds 24 hours. Similar exposure of parent and metabolite was observed on day 15 and 28 (except for the 25 mg dose), indicating PK steady state is reached on or before day 15. The PK profiles of AEE788 in rats and humans are similar, however, substantially less AQM674 is formed by rats (M/P ratio after a single intravenous or oral dose of 0.12 and 0.18, respectively).
Conclusion: After oral administration of AEE788 in pts with advanced cancer a significant amount of AQM674, the pharmacologically active metabolite, is rapidly formed by a saturable pre-systemic metabolic process. The serum concentration profile of AQM674 appears to reflect relative changes in parent, suggesting elimination of AQM674 is equal to or more rapid than that of AEE788.

413 POSTER

Pharmacokinetic/pharmacodynamic analysis of OSI-930, a novel selective tyrosine kinase inhibitor with anti-tumor activity

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We have recently identified a series of 2,3-substituted thiophenes with potent inhibitory activity against the tyrosine kinases Kit, KDR and PDGFRa/b, and OSI-930 has emerged from this series as an IND-track clinical candidate for use in tumor types dependent on these receptor tyrosine kinases including GIST, SCLC, renal cell carcinoma, colon carcinoma and glioblastoma. We have investigated the relationships between compound potency in cell-based assays in vitro, plasma exposure levels following oral dosing of OSI-930, the time course of target inhibition in vivo (KDR and Kit), and anti-tumor activity in tumor xenograft models. Thus, in the HMC-1 model, which expresses a constitutively activated form

of Kit, 50% inhibition of Kit phosphorylation is associated with a plasma concentration approximately equal to the IC50 for Kit inhibition in vitro, when measured in the presence of physiological concentrations of the plasma proteins albumin and alpha1-acid glycoprotein. Using the wild-type Kit-expressing H526 SCLC model we have found that higher plasma levels of OSI-930 are required to achieve a comparable level of inhibition of Kit phosphorylation. However, in both HMC-1 and H526 xenograft models, maximal tumor growth inhibition was observed at oral dose levels that maintained a high degree of inhibition of Kit phosphorylation (>90%) for the majority of the 24h dosing period, i.e. 50mg/kg q.d. in the HMC-1 model and 200mg/kg q.d. in the H526 model. The potential involvement of KDR inhibition in the anti-tumor activity of OSI-930 has also been investigated. Thus, OSI-930 potently inhibited VEGF-induced KDR phosphorylation in endothelial cells at concentrations that also inhibited angiogenic sprout formation from aortic ring explants. In addition, we have used the KDR-dependent mouse uterine edema model system to demonstrate that oral dosing of OSI-930 at 50mg/kg or above results in potent inhibition of KDR function in vivo, supporting a potential role for inhibition of KDR in the anti-tumor effects of OSI-930. The data suggest that anti-tumor activity of OSI-930 in mouse xenograft models is observed at dose levels that maintain a significant level of inhibition of Kit and KDR for a prolonged period. Therefore, prolonged inhibition of the molecular targets of OSI-930 in vivo may prove to be of therapeutic benefit in future clinical investigations of OSI-930 as a novel therapeutic agent.

414 POSTER

Correlation of mutations in EGFR with clinical outcomes in NSCLC patients treated with erlotinib

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Background: Erlotinib is a small molecule inhibitor of epidermal growth factor receptor (EGFR) that has demonstrated a statistically significant survival benefit in single agent treatment of unselected relapsed/refractory non-small cell small cancer (NSCLC) patients (ASCO 2004). *EGFR* mutations identified in a subset of NSCLC have been associated with sensitivity to gefitinib (Lynch et al. *NEJM* 2004; Paez et al. *Science* 2004). The prevalence of somatic mutations has been reported to be between 2% and 8% in unselected patients; mutations in exons 18 through 21 of *EGFR* were observed in 13 of 14 patients who responded to gefitinib and in none of the 11 patients who were treated and did not respond. The RAS pathway is also a target for somatic mutations in NSCLC. Mutations in *BRAF* and *KRAS* have been reported in 3% and 10–30% of NSCLCs, respectively, and have been associated with poor prognosis (Brose et al., *Cancer Res.* 2002;62:6997–7000; Silini et al. *Virchows Arch.* 1994;424:367–73).
In order to assess more accurately the prevalence of somatic mutations in NSCLC tumors and the influence of these mutations on patient outcome to erlotinib treatment, formalin-fixed paraffin-embedded tumor samples (FFPE) from patients treated with erlotinib in a phase **III** study were analyzed. Tribute, a phase **III** randomized trial conducted in the U.S, enrolled 1079 patients with previously untreated, advanced NSCLC to compare the survival of patients who received erlotinib administered concurrently with a regimen of carboplatin and paclitaxel (CP) (n=539) to patients who received CP alone (n=540). The erlotinib arm did not demonstrate a survival advantage (primary endpoint) over CP alone (ASCO 2004). Sample collection was optional in this study, and archival tumor tissue was available from a subset of the patients enrolled in the trial.
Methods: Mutational analysis of *EGFR*, *KRAS*, and *BRAF* in a subset of tumors from patients in Tribute was performed by DNA sequencing using fluorescent dye-terminator chemistry (Applied Biosystems, Foster City CA) of tumor cells isolated by laser capture microdissection (PixCell **II**, Arcturus). Descriptive summaries of duration of survival and objective response were produced for each of the categorical variables listed above for each treatment arm. These descriptive summaries consisted of the hazard ratio from unstratified Cox regression and Kaplan-Meier estimates of median time to the event.
Results and Conclusions: Baseline covariates were compared between the subsets of patients with and without tissue and no differences were observed. We have assessed the frequency and nature of mutations in this population, and have explored the effects of mutation status on survival, time to progression, and objective response rate survival. These data will be presented.

414A POSTER

Pharmacological properties and in vitro and in vivo antitumour activity of the potent and selective PI3 kinase inhibitor PI103

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PI3 kinase is strongly validated as an important therapeutic target, eg by loss of the *PTEN* tumour suppressor gene and by overexpression and mutation of the *PI3KCA* gene that encodes the p110α catalytic subunit. We previously reported the identification of the pyridofuropyrimidine PI103 as a nanomolar potent and selective inhibitor of the class IA p110α and β isoforms of PI3 kinase (Patel et al Proc AACR LB-247 2003). Here we describe the pharmacological properties and *in vitro* and *in vivo* antitumour activity of PI103. This agent exhibited an IC_{50} of around 1.5–3nM against p110α and p110β. PI103 exhibited growth inhibitory activity against a panel of human cancer cell lines and was more potent against the *PTEN* negative PC3 prostate tumour (IC_{50} 88nM) than against the *PTEN* positive HCT116 colon cancer line (IC_{50} 1 μM). The predominant effect on cells was a G1 cell cycle arrest. Rapid and extensive inhibition of Ser473 Akt phosphorylation was seen at concentrations around the IC_{50} for growth inhibition. Following 30mg/kg ip, PI103 was well distributed (Vz=150mL) but cleared relatively rapidly from the general circulation (Cl=120mL/hr). Tumour levels were above IC_{50} levels for 3hrs. Significant growth inhibitory activity of PI103 was seen in both PC3M and HCT116 human tumour xenografts using well tolerated doses of 30mg/kg ip twice daily. In addition to a direct antiproliferative effect on cancer cells, we have obtained data that suggest that the activity of PI103 may also be due in part to its inhibitory effects on invasion and angiogenesis. Thus PI103 was shown to inhibit the invasion of PC3M tumour cells and to block motility, invasion and proteolysis by human endothelial cells *in vitro*. PI103 showed significant inhibition of the OVCAR3 human ovarian cancer xenograft in vivo with a corresponding decrease in Akt phosphorylation, consistent with inhibition of PI3 kinase in the tumour. Additional potential pharmacodynamic and prognostic biomarkers have been identified by gene expression microarray analysis. In conclusion, PI103 is a potent and selective class 1A PI3 kinase inhibitor with promising pharmacological properties, including *in vitro* and *in vivo* antitumour activity consistent with the mechanism of action. Thus it exemplifies the considerable potential of agents derived from this series to be developed for clinical evaluation. Optimisation is now underway in collaboration with PIramed.

Cyclins and CDKs

415 POSTER

Discovery and evaluation of inhibitors of cyclin E2–CDK2 and cyclin B1–CDK1

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Cyclin-dependent kinase (CDK) complexes regulate the temporal progression of cells through the cell cycle. Accumulating evidence demonstrates that the deregulation of cyclin E expression and subsequent activation of the CDK2 catalytic subunit plays a critical role in the progression of multiple tumor types. To further understand the role of CDK complexes in tumor formation novel small molecule inhibitors that inhibited cyclin B1-CDK1 and cyclin E2-CDK2 complexes at low nM potency were evaluated in cell based assays and *in vivo* tumor xenograft models. A novel cell based assay that measures the intracellular level of Rb phosphorylation by flow cytometry was developed and used to screen CDK inhibitors. These compounds arrested cells in both G1 and G2, induced apoptosis and inhibited the phosphorylation of Rb at low sub-micromolar concentrations. CDK inhibitors were tested in *in vivo* tumor models and demonstrated potent activity against both colon and prostate tumor xenograft models. In addition, we demonstrate that the CDK inhibitors prevented Rb phosphorylation *in vivo* and induced tumor cell death in *in vivo* tumors models. The discovery and evaluation of novel CDK2 and CDK1 inhibitors may aid in delineating the potential role that these CDK complexes play in regulating tumor formation and progression.