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Head and neck squamous carcinoma cell lines exhibit an intact EGFR signaling pathway and variable response to EGFR agonist and antagonist

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Head and neck cancer is an epithelial malignancy expressing receptor tyrosine kinases of which the epidermal growth factor receptor (EGFR) has demonstrated clinical significance in this disease. Cetuximab (Erbitux) has resulted in survival benefits when added to standard radiotherapy in locally advanced head and neck cancer (Bonner et al. N Engl. J Med. 2006 Feb 9; 354(6): 567−78). We embarked on a study to examine a panel of head and neck cell lines, all of squamous cell histology, to determine the phosphorylation status of several signaling molecules of the EGFR pathway in relation to growth of the cells and the effect from EGFR agonist (transforming growth factor, TGF-α) and commercially available antagonist erlotinib (Tarceva).
Experiment 1: On day one, cells were seeded on 24 well plates with 10,000−40,000 cells per well. On day two, cells were treated in triplicate with either 5umol erlotinib or no treatment control (DMSO alone). On day five, cell count assays were performed to determine growth. This experiment was repeated two additional times to ensure reproducibility of the data. Experiment 2 consisted of 4 treatment arms of the complete cell line panel: erlotinib, TGF-α, erlotinib and TGF-α and no treatment control. Western blot analysis was performed on cell lysates for (phosphorylated) forms of ERK (pERK), AKT (pAKT), and RAF (pRAF), all downstream transducing proteins of the EGFR signaling pathway, as well as EGFR and human EGF receptor 2 (HER2). Degree of signal intensity was determined via phosphorimaging.
Results of the experiments were as follows: erlotinib was found to decrease all downstream phophorylated signal transducers of the EGFR pathway when compared to no treatment control in every cell line tested. In addition, inhibition of growth of cell lines was also consistently seen when compared with no treatment control. This growth inhibition in the presence of antagonist, however, was found to be independent of both EGFR/HER2 levels and the basal levels of the phosphorylated downstream transducers studied. Furthermore, it was also found that lower expression of HER2 in these cell lines correlated with greater inhibitory effect of antagonist upon the phosphorylated form of AKT, while higher levels of HER2 showed a lesser inhibitory effect of antagonist upon the prevalence of the phosphorylated form ($R^2 = 0.71$).
Variability of EGFR, HER2, and basal activated transducer levels as well as growth inhibition data between these cell lines suggest they are heterogeneous in their growth patterns, signaling pathways, and expression of receptors and transducers. This heterogeneity likely reflect biologic diversity of head and neck squamous cell carcinoma and supports the need for further study, data from which should improve our understanding of this disease as well as improve patient selection for this targeted therapeutic approach.

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Pharmacokinetics of Akt inhibitor NSC 728209 in the rat by LC/MS/MS method

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Purpose: NSC 728209 (OSU-03012, **I**) is a celecoxib derivative that was designed through molecular modeling to disrupt Akt signaling pathways by inhibiting PDK-1 with an IC_{50} of 5.0 μM and possesses antitumor activity against prostate and chronic lymphocytic leukemia. It was selected for preclinical development under the NCI RAID program and previously we reported the assay development and pharmacokinetics in mice (AACR04). The objective of the current study was to characterize the pharmacokinetics and oral bioavailability of **I** in the rat in comparison with that in the mouse and its metabolism in rat liver microsomes.
Methods: A sensitive and specific LC-MS/MS method previously developed for **I** in mouse plasma was adapted to rat plasma. The precursor/product ion pairs at m/z 461–404 for NSC 728209 and at m/z 446–429 for an analog NSC 728210 as the internal standard were used for the quantifications in rat plasma and microsomal extracts. Six Fisher 344 rats were given i.v. bolus of **I** and another six given p.o. doses, all at 10.0 mg/kg formulated in PEG400/ethanol/normal saline. Plasma pharmacokinetics were characterized and oral bioavailability determined based on the AUC method. Metabolism in rat liver microsomes and stability in rat plasma of NSC 728209 were also studied.
Results: Plasma concentration-time profile of **I** after an i.v. bolus dose reached a mean concentration of 4.4 μM at 5 min, which declined triexponentially with an initial half-life of 4.1 min, an intermediate half-life of 5.2 h and a terminal half life of 12.7 hr. Its pharmacokinetics are similar to those previously found in the mouse. The drug was detectable in plasma at 72 hr. After oral administration, the plasma level reached a mean C_{max} of 0.23 μM between 4–24 hr and was detectable at 48 hr. The total clearance in the rat was 37 ml/min/kg. The oral bioavailability of **I** was found to be 50.2% and in the mouse it was essentially complete at this dose. No evidence of metabolism was found in rat liver microsomes and the compound was stable in rat plasma at 37ºC, contrary to that in mouse plasma, which degrades with a half-life of 14 hr.
Conclusion: Plasma pharmacokinetics of **I** was similar between the mouse and the rat following i.v. dosing. The oral bioavailability shows species difference and no metabolism was found in rat liver microsomes or in rat plasma.
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Effect of the SRC tyrosine kinase inhibitor dasatinib in combination with erlotinib and in cells with acquired resistance to erlotinib

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Background: SRC tyrosine kinase proteins can regulate oncogenic processes such as cell growth, survival, invasion, and angiogenesis. We previously identified that lung cancer cells dependent on EGFR for survival demonstrate increased sensitivity to dasatinib, a SRC tyrosine kinase inhibitor (TKI). Here we evaluated the efficacy of dasatinib in combination with the EGFR TKI erlotinib in lung cancer cells with defined EGFR status and also examined the effect of dasatinib on lung cancer cells with acquired resistance to erlotinib.
Materials and Methods: Lung cancer cells with defined EGFR status and sensitivity to erlotinib were evaluated for the combination effect of erlotinib and dasatinib using cell viability assays. Combination effects were evaluated by median dose effect method. Cells with EGFR mutation with acquired resistance to erlotinib were used to evaluate the effect of dasatinib on cell viability, cell cycle, and apoptosis. pSRC expression was examined in these cells by western analysis.
Results: Using concentrations of gefitinib and dasatinib that result in concentration-dependent increases in apoptosis, our preliminary experiments suggest that dual EGFR/SRC inhibition additively or synergistically enhances apoptosis in PC9 lung cancer cells with EGFR mutation. We also examined the effect of dual EGFR/SRC TKI on lung cancer cells that do not have EGFR mutation but nonetheless show some degree of sensitivity to EGFR TKI. We identified synergy with erlotinib and dasatinib in both H292 and H358 cells at lower concentrations of both TKI while no effect was seen with either TKI in H441 cells in the dose range used. Both H292 and H358 cells show pSRC protein expression while H441 cells have low levels of detectable pSRC. Finally, lung cancer cells with EGFR mutation that are resistant to EGFR TKI were examined for the effect of dasatinib. These cells do not demonstrate significant amounts of apoptosis with dasatinib but they do undergo a dose-dependent G1 cell cycle arrest despite no observable effect on cell cycle with erlotinib.
Conclusions: The combination of erlotinib and dasatinib results in synergistic inhibition of viability and/or proliferation in lung cancer cells with dependence on EGFR for survival and/or growth. Resistance to erlotinib generally confers resistance to dasatinib although higher concentrations of dasatinib can induce cell cycle arrest, some degree of apoptosis, and reduced cell viability.

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Effect of the histone deacetylase inhibitor LBH589 against epidermal growth factor receptor dependent human lung cancer cells

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Background: The epidermal growth factor (EGFR) activates signal transduction pathways important in lung cancer cell growth and survival. Activating mutations in EGFR selectively activate STAT and Akt survival signaling pathways. Histone deacetylase (HDAC) inhibitors have been suggested to regulate signaling protein interactions via modulation of protein chaperone function. For these reasons, we evaluate the effect of the HDAC inhibitor LBH589 in lung cancer cells with defined EGFR status.
Materials and Methods: Human lung cancer cell lines with defined EGFR status and sensitivity to EGFR TKI were subjected to treatment with HDAC/Hsp90 inhibitor LBH589. Cytotoxicity assays (MTT) as well as assays specific for apoptosis and cell cycle changes were performed. Changes in cell survival were correlated with changes in signaling pathways known

to be important in cell survival including EGFR, Akt, Stat3, c-Src, PIM-1, and Bcl-2 proteins.
Results: Cells dependent on EGFR for survival demonstrated increased sensitivity to LBH589 and underwent apoptosis following exposure to these agents. LBH589 inhibits the binding of Hsp90 to EGFR. LBH589 selectively depleted proteins important in signaling cascades in cell lines harboring EGFR kinase mutations, such as EGFR, Stat3, and Akt. In addition, we found depletion of Stat3-dependent survival proteins including Bcl-xL, Mcl-1, and Bcl-2. Conversely, LBH589 had no effect on apoptosis in cells not dependent on EGFR for survival and no changes were identified in EGFR, Stat3, Akt, or Stat3-dependent survival proteins.
Conclusions: Based on these results, LBH589 can trigger apoptosis in EGFR-dependent lung cancer cells and depletes levels of key signaling cascades important in tumor survival.

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Modulation of the HSP90 co-chaperone AHA1 affects client protein activity and increases cellular sensitivity to the HSP90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG)

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AHA1 (Activator of HSP90 ATPase) is a co-chaperone of the ATP-dependent molecular chaperone HSP90, which is involved in the maturation and function of several oncogenic client proteins (Maloney A, Workman P. Expt Opin Biol Ther 2:3−24, 2002). HSP90, in association with its co-chaperones, operates as part of a multimeric complex driven by the binding and hydrolysis of ATP. The intrinsic ATPase activity of the human HSP90 has been shown to be significantly increased by AHA1 *in vitro*. Inhibition of HSP90 by the first-in-class HSP90 ATPase-inhibitor 17-AAG results in cessation of cell growth and the degradation of client proteins such as C-RAF and CDK4 via the ubiquitin proteasome pathway. Co-chaperones such as AHA1 and HSP72 have also been shown to be upregulated with 17-AAG treatment as a result of stress-induced transcription. As AHA1 is known to increase the ATPase activity of HSP90, we hypothesised that modulation of AHA1 expression could influence HSP90 activity and the cellular response to treatment with 17-AAG. We have previously shown that when AHA1 is knocked down using RNA interference, there is a significant ($P < 0.05$) increase in sensitivity to 17-AAG, as demonstrated by a 2–3 fold increase in detached cells (Holmes, J *et al*, Clinical Cancer Research 11(24 Suppl): 9157s, 2005). Further investigation into the effects of AHA1 modulation on cellular sensitivity to 17-AAG has shown that overexpression of AHA1 (3.5−10 fold) had no effect on sensitivity to 17-AAG. Using RNA interference and our AHA1 overexpression model, the role of AHA1 on HSP90 client protein activity has been investigated. When AHA1 protein expression was knocked down (~80%) by siRNA oligonucleotides there was no effect on HSP90 client proteins C-RAF, ERBB2 or CDK4. Similar results were obtained when AHA1 was overexpressed. Interestingly, however, MEK1/2 and ERK1/2 phosphorylation were decreased when AHA1 was knocked down with no change in the total protein levels. Moreover, overexpression of AHA1 resulted in an increase in phosphorylation of MEK1/2 and ERK1/2. These results would suggest that AHA1 may have a role in client protein activation, and modulation of AHA1 could be a therapeutic strategy to increase sensitivity to HSP90 inhibitors.

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Augmented growth inhibition of human NSCLC cells resistant to EGFR-tyrosine kinase inhibitor (TKI) by a combination of dual TKI of EGFR/VEGFR2 (AEE788) and mTOR inhibitor (RAD001)

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Background: EGFR-TKI such as gefitinib and erlotinib show anti-tumor activity in a subset of non-small cell lung cancer (NSCLC) patients having mutations of EGFR gene. However, clinical resistance to EGFR-TKI is observed in spite of the initial response. Recent work shows such resistance can be caused by a secondary mutation, T790M in the EGFR-TK domain. Several studies suggested the importance of the EGFR downstream kinases as potential drug targets. The aim of this study is to evaluate the efficacy of alternative small molecules which inhibit other targets than EGFR-TK, such as AEE788 and RAD001, for NSCLC cell lines. AEE788 is a dual TKI for EGFR and vascular endothelial growth factor receptor 2 (VEGFR2), while RAD001 is an inhibitor of the mammalian target of rapamycin (mTOR).
Methods: We used 3 human NSCLC cell lines, namely, A549, H1650 and H1975. A549 has wild type EGFR, H1650 harbors a deletion mutation in exon 19, while H1975 possesses double mutations at L858R and T790M, which account for sensitiveness and resistance to EGFR-TKI, respectively. We first treated these cells with AEE788 or RAD001 as a single agent, then tried combination of two agents and evaluated the effect on cell growth as well as the induction of apoptosis.
Results: AEE788, as a single agent, significantly reduced the proliferation of all cell lines dose-dependently. The degree of reduction, however, was much less in H1975 compared to other cell lines. The reduction was independent of inhibition of EGFR-TK activity as the status of p-EGFR was unchanged in H1975 after AEE788 treatment, suggesting the inhibition of other pathways, such as VEGFR by AEE788. RAD001 single-treatment also showed the growth inhibition of all cells with less effect in H1970 than in A549. The combined treatment with AEE788 and RAD001 showed no additional effect compared to AEE788 alone on growth inhibition in A549 and H1650. On the other hands, this combination resulted in effective and additional growth inhibition against H1975 and was related to induction of apoptosis.
Conclusions: AEE788 and RAD001 will be possible novel candidates for the treatment of NSCLC patients and will be especially useful to overcome the acquired resistance to EGFR-TKI when used in combination.

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Impressive anti-tumor activity of combined erbB1 and erbB2 blockade: a phase I and pharmacokinetics (PK) study of OSI-774 (Erlotinib; E) and Trastuzumab (T) in combination with weekly Paclitaxel (P) in patients (pts) with advanced solid tumors

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Background: Co-expression of erbB1 and erbB2 receptors confers a growth advantage in erbB2 overexpressing (+) cancers. Specifically, co-expression alters the normal rapid internalization and inactivation of erbB1, slows dissociation of the erbB1 ligand-receptor complex and degradation of the active receptor. Co-targeting both receptors together may offer a therapeutic advantage over targeting erbB2 alone, especially in T refractory solid tumors. A phase I and PK study was launched to determine the toxicity and recommended dose of continuous daily oral E in patients with erbB2 + cancers along with weekly P and T, especially since T improves survival in combination with chemotherapy.
Methods: Eligible pts were treated with weekly T IV (2 mg/kg/wk) along with weekly P (starting at 80 mg/m^2) and escalating doses of E po daily for 28-days. MUGA scans were performed at baseline and every 2 cycles (8 weeks). Two schedules – 3 out of 4 weeks and continuous weekly P and T were explored.
Results: 24 patients [breast (22), Colon (1), ovary (1)] have received 97 courses [median 2, range 1–13] in 5 cohorts. Doses of different drugs were E 50–150 mg (50 mg, 3 pts; 100 mg, 15 pts; 150 mg, 6 pts), P 80−90 mg/m^2 weekly for 3 out of 4 weeks and weekly T. Patients were women with median age 54 years [range 37−75] and PS 0 (5), PS 1 (17) or PS 2 (2). The proportion of patients positive for hormone receptors was 10/18. Thirteen patients received prior T and 8 patients had received T, including 6 patients who had previously received the combination TH. Also 13 patients had received Docetaxel (4 in combination with P). Dose limiting grade (gr) 3 diarrhea and gr 3 dermatitis was seen in 1 pt at 100 mg of E and 80 mg/m^2 of P. Other toxicities included gr 2 diarrhea, skin rash, fatigue, neutropenia and alopecia. Significant asymptomatic drop in LVEF was noted in 4 pt. One complete and four partial responses have been seen in pts with breast cancers, 2 of them have previously failed to T therapy and 3 had failed to taxanes. Three breast cancer pts experienced stable disease lasting 13, 11 and 6 courses, respectively. Preliminary PK data does not suggest a clinically relevant interaction between the 3 agents.
Conclusions: E combined with T and P provides a well-tolerated, targeted therapy with impressive anti-tumor activity in T-refractory breast carcinoma. Dose escalation was discontinued on the continuous dosing schedule as two patients experienced DLT. Expanded accrual is ongoing for the interrupted dosing schedule of 3 out of 4 weeks therapy at full doses of all three agents (MTD) to further characterize the toxicities.

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Targeting aberrant PI-3 kinase pathway signaling by dual inhibition of Akt and p70S6K

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The PI-3 kinase pathway is frequently dysregulated in cancer cells, and is implicated in multiple aspects of tumor growth and survival. In addition, resistance to many anticancer agents (including receptor tyrosine kinase inhibitors and genotoxic agents) has been attributed to failure to downregulate PI-3 kinase pathway signaling. Current inhibitors of this