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Pharmacodynamic markers for the choline kinase inhibitor MN58b in human breast cancer model by magnetic resonance spectroscopy

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Choline kinase (ChoK) is a cytosolic enzyme that catalyses the phosphorylation of choline to form phosphocholine (PC) which is involved in cell membrane synthesis. Elevated levels of PC and ChoK found in tumours are associated with cell proliferation and malignant transformation. Inhibition of ChoK with MN58b, a novel anticancer drug and putative competitive inhibitor, demonstrated an antiproliferative effect in human tumour xenografts (1).
The aims of this work were: a) to confirm the mechanism of action of MN58b; and b) to develop a robust and non-invasive surrogate marker for tumour response following MN58b treatment. We carried out an *in vivo* ^{31}phosphorus magnetic resonance spectroscopy (^{31}P MRS) study of MN58b (4mg/kg via ip for 5 days) and vehicle in MDA-231-MB human breast cancer xenografts. *In vitro* ^{31}P MRS, assays for ChoK activity and western blots for ChoK expression, were performed on tumour extracts.
A significant growth delay was observed in the MN58b-treated MDA-231-MB xenografts when compared with controls. *In vivo* ^{31}P MRS of the MDA-231-MB xenografts showed a decrease in the phosphomonoester/total phosphorus signals (PME/TotP) ($p<0.05$) and PME/β-NTP ratios ($p<0.05$) after 5 days of MN58b treatment. No significant changes were observed in the control group. *In vitro* ^{31}P MRS of extracts from MN58-treated tumours showed significant decreases in PC ($p<0.03$) when compared with controls. No changes in other phospholipid metabolites (phosphoethanolamine, glycerophosphocholine and glycerophosphoethanolamine) were observed. No significant changes in ChoK activity or expression were found in extracts from MN58b-treated tumours when compared with control. This is consistent with MN58b being a competitive inhibitor of ChoK.
Treatment with MN58b resulted in tumour growth delay and altered phospholipid metabolism *in vivo*. These MRS changes suggest inhibition of ChoK and are consistent with the mechanism of action of MN58b. The decrease of PC and PME may have potential as surrogate non-invasive pharmacodynamic markers for determining tumour response following treatment with MN58b or other ChoK inhibitors.
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Biological evaluation of a novel, synthetic pyrazole class of Hsp90 inhibitors

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The molecular chaperone, Hsp90 is an essential protein for maintaining the ATP-dependent folding and function of a multitude of key oncogenic client proteins, including c-Raf, ErbB2, Cdk4 and mutant p53. The inhibition of ATP binding at the N-terminal of Hsp90 ultimately leads to the degradation of the client proteins via the proteosome pathway. This provided a good strategy for generating novel Hsp90 inhibitors to overcome some of the limitations observed with the current Hsp90 inhibitor, 17AAG (17-allylamino-17-demethoxygeldanamycin), which is being evaluated in Phase I and Phase II clinical trials. The hit pyrazole compound CCT018159, which was discovered by high throughput screening at our Centre (Aherne et al., Proc. AACR 44, Abstract #4002) has led to synthesis of a series of closely related analogues, which have showed improved enzyme activity and cell potency. The ATPase and cellular IC_{50} values for CCT018159 (determined by malachite green assay and sulfohodamine B assay) were 6.4 and 4 μM, respectively. The derivatives of CCT018159 have demonstrated a 4–30 fold increase in enzyme potency and up to a 10-fold improvement in cellular potency in a range of tumour types, including colon and melanoma cell lines. The molecular signature of Hsp90 inhibition has been well defined (Maloney et al, Current Cancer Drug Targets, 3: 331–341, 2003); this includes upregulation of Hsp70 protein and downregulation of c-Raf, ErbB2 and Cdk4. This has been confirmed using western blotting and ELISA. These characteristic pharmacodynamic marker changes have been observed with the active analogues. Cell cycle analysis of these novel inhibitors using the dual-staining BrDU/Hoechst-propidium iodide method showed similar profiles to 17AAG and CCT101859. Apotosis confirmed by PARP cleavage has also been demonstrated in HCT116 treated colon cancer cells. 17AAG activity has been shown to be dependent on the quinone reductase enzyme DT-diaphorase and the multidrug transporter, P-glycoprotein (Kelland et al., J. Natl. Cancer Institute, 91:1940–1949, 1999). Cellular activity of these pyrazole analogues is not significantly altered in a BE colon cell line transfected with the DT-diaphorase gene, *NQO1* or in a P-glycoprotein positive cell line. Pharmacokinetic properties of this class of Hsp90 inhibitors look promising (Smith et al., Clin. Cancer Res., *9:* 239, 2003) and solubility is much favourable than 17AAG. Phase I combination trials of 17AAG and docetaxol are ongoing in the USA, and a phase II clinical trial of 17AAG alone in melanoma is scheduled. *In vitro* combination studies with 17AAG or CCT018159 and temozolomide or dacarbazine in a melanoma cell line, SKMEL5 are underway. In summary, this series of pyrazole compounds has contributed significantly to the lead optimisation program aimed to identify developmental clinical candidates.
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HER2 imaging as a non-invasive pharmacodynamic marker of Hsp90 inhibition

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17-allylamino-geldanamycin (17-AAG) is the first Hsp90 inhibitor to be tested in clinical trial. This agent causes the ubiquitination and proteasomal degradation of HER2 and other Hsp90 client proteins. To date, we have treated 85 patients with various cancers on two clinical trials of 17-AAG alone and in combination with docetaxel. One obstacle in the clinical development of this class of agents is the inability to quantitatively assess as a function of time the effect of the drug on Hsp90 function in patients. While the optimal correlative assay is pre- and post-treatment collection of tumor tissue for analysis of treatment induced changes in relevant Hsp90 client proteins, in only a small fraction of the patients treated on these protocols was tumor tissue accessible for biopsy. As an alternative, we have sought to determine changes in chaperone and client protein expression in surrogate tissues such as skin or peripheral blood lymphocytes. In these studies, post-therapy increases in Hsp70 in peripheral blood lymphocytes (PBL) have been observed in all patients treated with 17-AAG at or above 110 mg/m^2 while declines in Raf-1 and Akt have been seen in a minority of patients treated at doses of 17-AAG above 160 mg/m^2. As an alternative, we have developed a method for the non-invasive quantification of HER2 protein expression in tumors. HER2 is an Hsp90 client protein and 17-AAG inhibits HER2 signaling by inducing its proteasomal degradation. We labeled an F(ab')$_2$ fragment of Herceptin (trastuzumab) with ^{68}Ga, a positron emitter, which allows for the sequential non-invasive quantitation of HER2 expression using Positron Emission Tomography (PET) imaging. The rapid elimination of this radiotracer from the blood pool allowed for serial determinations of HER2 expression pre- and post treatment with 17-AAG. Imaging results correlated with direct determinations of tracer uptake by gamma counter and with changes in HER2 protein expression by immunoblot. This technology has immediate clinical applicability as a pharmacodynamic marker in the ongoing clinical trials of Hsp90 inhibitors. It also highlights the potential utility of molecular imaging technologies in the translation of novel targeted therapies from the laboratory to patients.

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Restoration of PTEN-expression in tumor cells causes them to depend on EGFR for suppression

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Activation of EGFR-mediated signaling pathways in epithelial cells stimulates their proliferation and desensitizes them to apoptotic stimuli. Tumor cells with mutated PTEN are EGFR-independent, however, and are insensitive to selective inhibitors of EGFR. Restoration of PTEN-expression sensitizes these tumors to EGFR inhibitors. We introduced a tet-inducible PTEN into the PTEN-deficient, EGFR-overexpressing breast cancer cell line MDA-468 in order to study the mechanism of this effect. In this cell line, PI3 kinase and Akt kinase are activated, EGFR independent and insensitive to Iressa, an inhibitor of EGFR tyrosine kinase. Induction of PTEN expression inhibits Akt kinase and causes a slowing of growth. Expression of wt PTEN sensitizes these cells to EGFR inhibition by Iressa. Growth inhibition is not due to cell cycle arrest but to synergistic induction of apoptosis. This synergy is not due to further inhibition of Akt kinase

by Iressa; Akt activity is maximally inhibited by induction of PTEN and PI3 kinase is not regulated by EGFR in these cells. Instead the synergy seems to be secondary to inhibition of two parallel pathways that both inhibit the function of the proapoptotic protein Bad. Bad is phosphoryated on serine 112 and serine 136 in these cells. Serine 112 phosphorylation is EGFR-MEK-MAP kinase dependent, whereas phosphorylation of serine 136 is sensitive to inhibitors of PI3 kinase, Akt kinase signaling. Bad function is inhibited and apoptosis is induced maximally only if both serines are dephosphorylated in response to inhibitors of both pathways. Furthermore, conditional expression of PTEN function results in inhibition of Akt phosphorylation in MDA-468 xenografts and a delay in tumor growth. These findings suggest that EGFR activation is not required for cell survival in cells with PTEN deficiency and that selective EGFR inhibitors will be ineffective in such tumors. However, if PI3 kinase, Akt kinase signaling is inhibited, cells become dependent for survival on EGFR driven, Akt independent, inactivation of Bad. Inhibition of EGFR and PI3 kinase signaling by combinations of drugs may be useful for the treatment of such tumors.

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Clinical and molecular features of neuroblastoma tumors that can be targeted through c-Kit

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Background: In neuroblastoma (NB) recent reports show that the selective inhibition of c-Kit signaling by STI571 (Gleevec) is associated with significant tumor growth inhibition in *in vitro* and *in vivo* preclinical models (Vitali R et al, 2003; Beppu K et al 2004). This study was aimed at investigating in a large clinical series of NB primary tumors the clinico-biological characteristics of that subset which utilizes the SCF/c-Kit pathway and may thus be responsive to selective inhibitors.
Material and Methods: Primary tumor site samples were obtained at diagnosis from 168 untreated children with NB. Expression of mRNA and protein for c-Kit and its ligand Stem Cell Factor (SCF) was determined by Northern blot and immunohistochemistry, resp. In selected cases sequencing of c-Kit exon 11 was also carried out in order to identify possible mutations. MYCN amplification and allelic loss for 1p36 (1p36 LOH) were evaluated by Southern blot and demonstrated in 27 and 36 tumors, resp.
Results: Expression of mRNA and protein for c-Kit was detected in 22% and 13% of tumors, resp. Immunostaining was confined to neoplastic neuroblasts. Expression of mRNA and protein for SCF was documented in 31% and 28% of tumors, resp., with 66% of the c-Kit-positive tumors also expressing SCF. Mutations in exon 11 of the c-kit gene were not found in the 9 c-Kit-positive and 9 c-Kit-negative tumors that were analyzed. Expression of c-Kit correlated with advanced stage (3 and 4), MYCN amplification and 1p36 LOH ($p<0.001$). Expression of SCF correlated with adrenal primary ($p<0.05$), MYCN amplification and 1p36 LOH ($p<0.001$). Overall survival (OS) probability was 17% in c-Kit-positive cases vs. 68% in c-Kit-negative, 43% in SCF-positive cases vs. 78% in SCF-negative ($p<0.001$). Using Cox multiple regression analysis however neither c-Kit nor SCF expression were independently associated with a shorter OS.
Conclusions: The SCF/c-Kit pathway is expressed overall in 20% of NBs, but this represents 60% of the most aggressive tumors (i.e., metastatic disease with unfavorable clinical, histological and molecular features). At present these children cannot be cured by high-dose cytotoxic chemotherapy. Preclinical studies suggest that these patients may benefit from the administration of selective inhibitors of c-Kit, and human clinical trials are therefore now warranted.
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C-fos mRNA levels predict response to Iressa therapy

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The response to EGFR inhibitors has been measured using clinical and radiological parameters. In this abstract we present the development of a molecular response assay, which predicts sensitivity to EGFR blockade *in vitro*, and tumor response to therapy *in vivo*.

Human carcinoma cell lines (A431, HuCCT1, HN11, 29, CAL29 and Hep2) were grown under standard tissue culture conditions. The cell lines were stimulated for 1 hour with recombinant EGF, in presence or absence of inhibitor (Iressa). 2 groups of 5 nude athymic mice were injected (2 tumors per mouse) with $5x10^6$ cells, and treated during 14 days (vehicle or Iressa 150 mg/kg, intraperitoneally). Fine needle aspiration biopsy of tumors was performed according to standard cytopathologic practice at 1, 7, and 14 days of therapy. mRNA was obtained for quantitative PCR analysis. The levels of c-fos expression were normalized to 3 house-keeping genes (HPRT, SDHA and Ubiquitin).
3 cell lines showed markedly elevated levels of c-fos mRNA in response to EGF stimulation (18 to126-fold), which was completely inhibited by the addition of Iressa. Two cell lines (HUCCT1 and Hep2) showed no increase in c-fos levels. None of the cell lines showed a decrease of c-fos levels with inhibitor alone at 1 hour. When grown over 72 hours, A431 showed a 6-fold decrease in c-fos levels in presence of Iressa (HuCCT1 showed no change).
Iressa induced tumor growth arrest in A431 xenografts, and a decrease in c-fos levels was documented in the treated vs. the control arm, showing a significant correlation with response to therapy. The c-fos levels were: baseline at d1: 7.3 (std.error 4.9), d7: 3.6 (1.2), d14: 3 (1.6). The c-fos levels in the control group were 4.5-fold higher than in the treated group: baseline: 3.9 (1.2), d7: 14.3 (3.3), d14: 14.1 (2.8). There were no significant differences between treated and control groups at baseline. HuCCT-1 xenografts showed no difference between treated and control groups in terms of tumor growth and c-fos levels.
C-fos quantitation can be used in an in-vitro EGF stimulation assay for assessment of EGFR blockade by Iressa. Importantly, the assay is useful in predicting lack of response in cases of activating mutations downstream of EGFR proximal phosphorylation events.
C-fos mRNA levels predict tumor response to Iressa therapy in a xenograft model in both sensitive and resistant cell lines. We are in the process of validating the assay in prospective trials in patients receiving Iressa.

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Constitutive activation of Akt/protein kinase B in gastric cancer: its correlation with lower invasiveness and better prognosis

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Background: Gastric cancer is one of the most common malignancies worldwide and a major cause of cancer death in Asia. Thus, it is important to identify genetic alterations that allow malignancy and prognosis of gastric carcinoma to be estimated. Phosphatidylinositol-3 kinase/Akt (protein kinase B) pathway has been reported to promote cell proliferation, survival and tumor progression. However, the role of Akt in the biology of gastric cancer has not been well studied. We sought to investigate the expression of Akt phosphorylation in human gastric carcinomas and its biological significance.
Material and Methods: The expressions of Akt and pAkt were evaluated immunohistochemically in 329 consecutive gastric carcinomas using the tissue-array method, and the associations between pAkt and clinico-pathologic features were assessed. Survival curves were estimated using the Kaplan-Meier product-limit method, and the significance of differences between the survival curves was determined using the log-rank test. We also performed Western blot analysis and cell growth assay using human gastric cancer cell lines which were transduced with retroviral vectors containing constitutively active Akt (CA-Akt) or kinase-dead Akt (KD-Akt).
Results: Akt expression was detected in 74% of the tumors and pAkt expression in 78%. Akt phosphorylation was highly expressed in the early stage gastric carcinomas ($p=0.01$). We also found an inverse association between Akt activation and lymphatic invasion ($p=0.01$) or lymph node metastasis ($p=0.008$). Patients with pAkt-positive carcinomas showed significantly better survival than those with pAkt-negative carcinomas ($p=0.0002$). An evaluation of combined expressions revealed that the group with pAkt-positive plus LN-negative had a better prognosis than the other cases ($p<0.0001$). *In vitro* study showed that constitutive activation of Akt promoted cell growth in gastric cancer cells.
Conclusions: pAkt, which is highly expressed in the early stage of gastric carcinoma, is significantly correlated with cell growth, nodal status and better outcome. These findings suggest that Akt activation may be used as an independent prognostic marker in gastric cancer.