**1154** POSTER

### Quantification and Localisation of Activated HER2 and EGFR Using High Content Analysis (HCA)

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**Background:** Epidermal growth factor receptor (EGFR) and Human EGFR 2 (HER2) are members of the ErbB family of receptor tyrosine kinases (TKs). Activation of EGFR and HER2 signalling pathways play a role in the initiation and progression of breast cancer. EGFR, is overexpressed in up to 60% of breast tumours and is characteristic of highly aggressive molecular subtypes of breast cancer with basal-like and BRCA1 mutant phenotypes. HER2 is overexpressed in up to 30% of breast cancers and plays an important role in regulating cell survival, proliferation, angiogenesis, invasion and metastasis. Tumour cells overexpressing both EGFR and HER2 exhibit aggressive tumour cell growth. Several targeted therapies against both EGFR (gefitinib/erlotinib) and HER2 (trastuzumab/lapatinib) are currently being used in patient treatment protocols. High Content Analysis (HCA) is a powerful screening tool which is used to quantify changes in protein expression and track changes in protein localisation. To elucidate the effect of the aforementioned targeted agents on signalling we have developed HCA assays to quantify changes in total receptor expression, cleaved receptor localisation and phosphorylation for both EGFR & HER2.

**Materials and Methods:** A panel of antibodies for total and activated EGFR and HER2 were validated by Western blot analysis. Fixation protocols using paraformaldehyde, methanol and trichloroacetic acid were compared. Permeabilisation and immunofluorescence staining procedures were also optimised and validated. HCA algorithms were developed to quantify both staining intensity and localisation.

**Results:** After extensive evaluation of a range of different fixation/permeabilisation protocols it was determined that fixation techniques that utilise TCA are superior to the more commonly used PFA or methanol for the quantification of total and phosphorylated EGFR/HER2 localization. This may be due to the mechanism of denaturing proteins and the inhibition of phosphatases by TCA. As a result we have established a robust cell based model for the study of EGFR and HER2 activation/inhibition and localisation in intact cells.

**Conclusions:** This method is superior to other standard methods such as Western blot as it allows for the simultaneous assessment of the phosphorylation status and sub-cellular distribution of multiple targets at the single cell level.

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### Synergistic Interaction Between MEK and MTor Inhibitors in Cancer Cells With PTEN Loss

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**Background:** PTEN is a lipid phosphatase counteracting the activity of the PI3K pathway, one of the most critical cancer-promoting pathways. PTEN mutations and deficiencies are prevalent in many types of human cancers and also associated with poor prognosis and therapeutic resistance. MAPK is another key cellular network that works independently, in parallel, and/or through interconnections with PI3K to promote cancer development. Here we investigated whether a strategy combining MEK/ERK and mTOR inhibition may be effective in preclinical models of human cancers and the role of PTEN loss in determining sensitivity/resistance to single and combined pathway inhibitors.

**Materials and Methods:** We employed *in vitro* assays, including cell proliferation assays, cell cycle analysis, annexin V binding assay, WB, and ELISA assay to determine functional and molecular drug effects. Pharmacologic interactions between mTOR and MEK inhibitors were analyzed by conservative isobologram analysis using a fixed-dose ratio experimental design.

**Results:** In cell lines with wt-PTEN (MDA-MB361 − breast; NCI-H1975 − lung; M14 − melanoma), simultaneous inhibition of both mTOR and MEK achieves synergistic effects at suboptimal concentrations, but becomes frankly antagonistic in the presence of complete inhibition MEK-to-ERK signaling (combination indexes − CI − 4.5, 90, and 200, respectively). This observation led to the identification of a novel general crosstalk mechanism, by which inhibition of constitutive MEK signaling restores PTEN expression and inhibits downstream signaling, thus bypassing the need for double pathway blockade. Consistent with this model, in cancer cell lines with PTEN gene loss combined mTOR and MEK blockade showed strongly synergistic effects in terms of cell growth inhibition (BT549 − breast − CI: 0.281; H1650 − lung − CI: 0.033; WM115 − melanoma − CI: 0.045).

**Conclusions:** Our results suggest that combined mTOR and MEK inhibition has strongly synergistic effects in cancer models with PTEN gene loss. This notion may be helpful in selecting appropriate cellular contexts for the design of rational therapeutic strategies based on the combined inhibition of the RAF/MEK/ERK and PI3K/AKT/mTOR pathways.

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### Synergistic Activity of 'Vertical' Combinations of Agents Targeting the RAF/MEK/ERK Cascade as a Therapeutic Strategy in Human Tumours

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**Background:** ATP-competitive, BRAF-selective, kinase inhibitors have potent antitumour effects in mutant BRAF(V600E) tumours and are clinically effective in malignant melanoma; however, under certain conditions they paradoxically activate the MEK/ERK kinase module downstream. In addition, different tumour models exhibit variable responses to MEK inhibition and MEK blockade may induce compensatory signaling through both upstream pathway elements (RAF) and parallel pathways (PI3K/AKT/mTOR).

**Methods:** We set out to define molecular and functional effects of single and combined BRAF (GSK2118436A, BRAF-I) and MEK (GSK1120212B, MEK-I) inhibition, using WB analysis to dissect signaling and fixed dose-ratio experimental design (1000:1) to assess functional synergism by conservative isobologram analysis.

**Results:** In A549 lung adenocarcinoma (KRAS G12S), BRAF-I (10 mM) induces hyperphosphorylation of MEK, ERK and p90RSK, while MEK-I (10 nM), alone or in combination with BRAF-I, potently offsets MAPK activation. Combined BRAF-I and MEK-I suppress malignant growth and survival at 72 h with highly synergistic effects in the A549 lung adenocarcinoma (KRAS G12S), HCT116 colon carcinoma (KRAS G13D), and MIAPACA pancreatic adenocarcinoma (KRAS G12V) models (combination indexes − CI − 0.077, 0.001, and 0.047, respectively). Conversely, in other lung cancer models with Q61H and G12C KRAS mutations (H460 and Calu-1, respectively) or wt-KRAS (Calu-3) the combination of BRAF-I and MEK-I produced modestly additive (H460, CI 0.8) to highly antagonistic antitumour effects (Calu-1 and Calu-3, CI $2x10^4$ and 4.4, respectively). Similar results were obtained in melanoma models: in the M14 model (mut-BRAF/wt-NRAS), both BRAF-I and MEK-I had pronounced growth inhibitory effects as single agents, but were frankly antagonistic in combination; in the ME1007 model (wt-BRAF/mut-NRAS), MEK-I, but not BRAF-I, effectively inhibited cell growth but there was no synergistic effect with the combination, despite the fact that BRAF-I induced MEK/ERK hyperactivation.

**Conclusions:** Overall, our data indicate that combined inhibition of multiple signaling elements along the RAF/MEK/ERK pathway results in strongly synergistic growth inhibition, particularly in tumours with specific KRAS mutations. Additional studies to better define genetic determinants of sensitivity/resistance and molecular mechanisms of therapeutic synergism of combined BRAF-I and MEK-I are currently ongoing.

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### Growth Inhibition of Mammalian Target of Rapamycin (MTOR) in Malignant Pleural Mesothelioma

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**Background:** Malignant pleural mesothelioma (MPM) is associated with poor prognosis and despite recent advances in chemotherapy; the median survival is still approximately 12 months following treatment with Pemetrexed and Cisplatin. Currently, there are no standard second line treatment options for advanced MPM. Activation of the PI3K/AKT/MTOR pathway has been shown to play an important role in MPM. MTOR can be assembled into two different complexes (MTORC1 and MTORC2). MTORC1 is sensitive to the inhibitory effects of Rapamycin, whereas MTORC2 is Rapamycin insensitive. In this study we aimed to analyze the cytotoxic effect of MTORC1 inhibition and the effect of combined MTORC1 and MTORC2 inhibition in MPM cell lines using the MTS cell proliferation assay.

**Materials and Methods:** The MPM cell lines MSTO-211H, NCI-H2052 and NCI-H2452 and the lung cancer cell line A549 were incubated with the MTORC1 inhibitor Rapamycin (Tocris, cat no 1292), and the combined MTORC1/MTORC2 inhibitor Ku0063794 (Tocris cat no 3725), at various dilutions for 72 hrs in a 96 well plate. At the end of 72 hrs the 96 well plate was analysed for cell viability using the MTS assay (Promega, cat

no G3580). In each experiment 6 replicate wells were used for each drug concentration and the experiment was repeated 3 times. The average of the three experiments was taken, plotted onto a graph and IC50 values calculated.
**Results:** Rapamycin showed significant growth inhibition in the NCI-H2052, NCIH2452 and A549 cell lines with IC50 values of 675pM, 565pM and 620pM respectively, but not in the MSTO-211H cell line up to a maximum concentration of 1μM. Similarly, Ku0063794 demonstrated significant growth inhibition in the NCI-H2052, NCIH2452 and A549 cell lines with IC50 values of 10 nM, 135 nM and 100 nM respectively, but not in the MSTO-211H cell line up to a maximum concentration of 1 μM.
**Conclusions:** This study demonstrates that inhibition of MTORC1 alone or combined inhibition of MTORC1 and MTORC2 may be an important therapeutic strategy in patients with MPM.

**1158** POSTER

**Inhibition of Epidermal Growth Factor Receptor in Malignant Pleural Mesothelioma**

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**Background:** Advanced malignant pleural mesothelioma (MPM) is associated with poor prognosis with median survival of approximately 12 months, despite recent advances in chemotherapy. The incidence of MPM varies from country to country, but is on the rise in most parts of the world. Great Britain, Australia and Belgium have the highest annual crude incidence rates of 30 cases per million population. Immunohistochemical studies have shown that Epidermal Growth Factor Receptor (EGFR) is over expressed in 44 to 97% of MPM tissue samples. In this study we aimed to determine the cytotoxic effect of EGFR inhibition in MPM cell lines using the MTS cell proliferation assay.
**Materials and Methods:** The MPM cell lines MSTO-211H, NCI-H2052 and NCIH2452 and the lung cancer cell line A549 were incubated with the anti-EGFR monoclonal antibody, Cetuximab (provided by Merck KGaA, Germany), and EGFR tyrosine kinase inhibitor, Gefitinib (Tocris, cat no 3000), at various dilutions for 72 hrs in a 96 well plate. At the end of 72 hrs the 96 well plate was analysed for cell viability using MTS assay (Promega, cat no G3580). In each experiment 6 replicate wells were used for each drug concentration and the experiment was repeated 3 times. The average of the three experiments was taken, plotted onto a graph and IC50 values were calculated.
**Results:** Cetuximab demonstrated significant growth inhibition in the MSTO-211H cell line with an IC50 value of 1.6 μM. No significant growth inhibition was seen in the NCI-H2052, NCI-H2452 and A549 cell lines at the maximum concentration of 1.75 μM, which was more than the maximum achievable serum concentration (1.57 μM) in Phase 1 studies. Similarly Gefitinib demonstrated significant growth inhibition in the MSTO-211H cell line with an IC50 value of 1.6 μM. The NCI-H2052, NCI-H2452 and A549 cell lines showed growth inhibition at much higher concentration with IC50 values of 3.7 μM, 6 μM and 13 μM respectively, which were more than the maximum achievable serum concentration (3.1 μM) in Phase 1 studies.
**Conclusions:** Our study suggests that anti-EGFR therapy may be effective in a select subset of patients with MPM. Despite there being significant over expression of EGFR receptors in MPM, various resistance mechanisms may exist resulting in resistance to anti-EGFR therapy.

**1159** POSTER

**Modulating Effect of Microenvironment Factors on Hormone Therapy of Breast Cancer**

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**Background:** The most effective drugs for breast cancer are antiestrogen supplements such as tamoxifen (TAM). Loss of cell sensitivity to TAM may be associated with decreasing of number of steroid receptors in breast tumours. Indeed, estradiol, pro-inflamatory cytokines and IFN-γ may modulate ER expression of, but they are activated in different stages of malignization process, that's why its influence on receptor status will also have differences. Thus it will be important to investigate the impact of factors of cell microenvironment on ER expression, proliferation, apoptosis and cell cycle in MCF-7 cells on models of different breast cancer stages.
**Materials and Methods:** MCF-7 cells were cultured under standard conditions. For cocultivation a cell line MT-4 (human cell chronic lymphocytic leukemia) were used. Recombinant IFN-γ was added at a concentration of 10 U/ml, TAM − 100 nM, E2 − 10 nM, condition medium (C-medium) from T-lymphocytes − 1:1 with culture medium. Cell survival was determined by MTT test. The distribution of the cell population between cell cycle stages was measured using flow cytometry. Expression of ER and EGF-R was visualised by immunocytochemistry (DAKO, USA).
**Results:** Our results have indicated that recombinant IFN-γ has a cytostatic effect in comparison with a cytotoxic effect of TAM and a proliferative effect of estradiol. Increasing of cell number was shown for C-medium with E2, IFN-γ with E2, TAM with E2 and IFN-γ with TAM in suspension fraction. Decreasing of the cell number was demonstrated for IFN-γ, TAM, C-medium with TAM in suspension fraction. In adhesion fraction TAM, TAM with E2 and TAM with C-medium decreased the number of alive cells. IFN-γ, C-medium, IFN-γ with TAM and TAM with C-medium decreased cell number in S phase. IFN-γ and TAM increased cell number in G2/M phase, C-medium from T-lymphocytes and IFN-γ with TAM increased cell number in G0/G1 phase. In adhesion fraction apoptosis was stimulated by IFN-γ with E2, TAM, C-medium with E2. IFN-γ and C-medium from T-lymphocytes stimulated ER expression in MCF-7 cells.
**Conclusion:** Perhaps TAM has become a first agent for target therapy. Thus, our data demonstrated that cell microenvironmental conditions (hormonal and humoral) have a strong influence on ER expression in breast cancer cell and as a result modulate sensitiveness to antiestrogen therapy. Combination antiestrogen therapy with balanced approach IFN-γ, activated T-cells and level of E2/Pr may has commulative effect in antitumour treatment.

**1160** POSTER

**Induction of Hypoxia by Vascular Disrupting Agents and the Significance for Their Combination With Radiation Therapy**

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**Background:** Targeting tumour vasculature is an increasingly popular therapeutic approach. The resulting vascular changes may also alter the tumour microenvironment and could influence conventional therapies given in combination. We investigated this issue using various vascular disrupting agents (VDAs) currently in clinical evaluation.
**Materials and Methods:** Restrained non-anaesthetised mice with 200 cubic mm foot implanted C3H mammary carcinomas were used. The VDAs were combretastatin A-4 phosphate (CA4P), its analog Oxi4503, and 5.6-dimethylxanthenone-4-acetic acid (DMXAA); they were dissolved in saline and intraperitoneally injected at doses of 250 (CA4P), 50 (Oxi4503), and 20 (DMXAA) mg/kg. Tumour oxygenation was determined using the Eppendorf polarographic electrode; the endpoint being the percentage of oxygen (pO2) values below 5 mmHg. Tumours were also locally irradiated (230 kV x-rays) in either single or fractionated (10 fractions in 12 days) schedules. The percentage of mice in each treatment group with local control at 90 days was recorded and the TDC50 values (radiation dose to control 50% of tumours) estimated from full radiation dose response curves. A Student's t-test (Eppendorf) or Chi-squared test (TCD50) were used for statistical analysis (significance level of $p < 0.05$).
**Results:** The average (with 1 S.E.) percent pO2 values below 5 mmHg was 45% (40−50) for control tumours. After injecting the VDAs, this significantly increased to around 90%. The TCD50 value (with 95% confidence intervals) for single radiation treatments was 53 Gy (51−55). Injecting VDAs immediately or within a few hours after irradiating significantly reduced this value to 46 Gy (42−49) and 45 Gy (41−49) for CA4P and DMXAA, respectively. This enhancement was lost if CA4P or DMXAA were injected immediately prior to irradiation. With Oxi4503, the TCD50 values were around 41 Gy (38−45) regardless of the time interval or sequence of the treatments. The TCD50 value for fractionated radiation was 76 Gy (73−9). Irradiating tumours and then injecting CA4DP or Oxi4503 after 5 and 10 radiation fractions significantly reduced the respective TCD50s to 66 Gy (62−69) and 67 Gy (63−71).
**Conclusions:** VDAs increase tumour hypoxia that can reduce the efficacy of radiation given shortly after drug treatment. However, hypoxia is not a problem if the VDA is given after irradiating or one uses a VDA like Oxi4503 that is also cytotoxic and thus can kill any induced hypoxic cells.

**1161** POSTER

**Evaluation of Drug Response of Trastuzumab Treated Cultivated Breast Cancer Tissue Slices**

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**Background:** The aim of this study was to advance the previously developed preclinical model of cultivated cancer tissue slices to the application of therapeutic antibodies such as Trastuzumab (Herceptin®) thus allowing detailed drug testing in a natural tumour microenvironment.