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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