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The effect of carbohydrate- and fat-restricted diet on B16F10 melanoma model in C57BL6 mice: focus on tumor growth inhibition and its underlying molecular target signaling pathways

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Background: In cancer metabolism, cancer cells have higher aerobic glycolysis than that of normal cells even when oxygen is present. Increased glucose uptake and glycolysis in cancer cells have been shown to correlate with poor prognosis, increased invasiveness and metastatic potential in numerous cancers. Recent evidence reported that dietary restriction of carbohydrate and energy supplies may influence various cancer cells metabolism and their growth delay. However, the effect of those nutrient restrictions and their underlying signaling mechanisms are not well understood.
In our study, we tested whether restriction of dietary carbohydrate and fat (CFRD), without energy restriction relative to comparison diets, would slow tumor growth and reduce the molecular survival signaling pathway in B16F10 mice melanoma which is generally chemoresistant and carries poor prognosis.
Material and Methods: Male C57BL6 mice were randomly divided into two groups (n = 15/group) and fed experimental diet as follows: CFRD (20% carbohydrate, 2% fat, 78% protein, including ingredients supplementation) and regular diet as control. Following a preliminary feeding period for one week, mice were subcutaneously injected with 1×10^6 B16F10 cells and tumor volumes were measured for 3 weeks and sacrificed. Tumor tissues were excised for western blot and microarray analysis for the evaluation their molecular mechanisms of action and differences in gene expression.
Results: CFRD fed mice had decreased tumor volume and prolonged survival by up to 47% and 33.4%, respectively. In order to examine the mechanism of action of CFRD in the melanoma xenograft model, the expression pattern of several intracellular signaling molecules related to energy metabolism within the excised melanoma tissues were evaluated. Higher expression levels of hexokinase (HK)-II, PI3K/*p*-Akt and mammalian target of rapamycin (mTOR) related signaling were significantly attenuated by CFRD. We also observed increased expression of AMP-activated protein kinase (AMPK) related signaling proteins in CFRD mice tumor compared with control group. In addition, melanogenic-related genes and proteins such as DEK, MITF and TYRP-1 were decreased in CFRD mice.
Conclusions: Taken together, these findings suggested that CFRD not only delays or inhibits tumor growth, but also affects melanoma cell metabolism and survival signaling pathways in the both levels of protein and gene expression, resulting in the modulation of HK-II, AMPK, mTOR, Akt and melanogenic-related protein, which plays a key role in the survival of the melanoma cells. Overall, our study provides a rationale and experimental basis for using a combination of CFRD and chemotherapeutic drugs to improve treatment of melanoma cancers.

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Evaluation of the antitumor activity of pemetrexed in combination with the Chk1 inhibitor LY2603618

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The antifolate pemetrexed (Alimta®, PMX), which targets thymidylate synthase (TS), is currently approved by FDA and EMEA for the treatment of selected indications of advanced or metastatic NSCLC of nonsquamous histology. Inhibition of cellular de novo thymidine nucleotide synthesis is known to induce cell cycle arrest and checkpoint activation. The combination of PMX with the checkpoint kinase 1 (Chk1) inhibitor LY2603618 is currently undergoing phase 2 clinical trial. Here we report its preclinical activity in NSCLC tumor models. Treatments of subcutaneous xenografts implanted in female athymic nude mice via intraperitoneal route were initated when tumor volumes averaged 100 mm^3. Antitumor effects were assessed on day 67 using ratios of the average tumor volumes of treated (T) to vehicle control (C) group in percent (=100X[T/C]). In the H2122 xenograft model, PMX alone at 100 mg/kg given at q3dx15 caused T/C = 73%. PMX at this dose and schedule combined with LY2603618 at 45 or 90 mg/kg given 24 h after each PMX injection resulted in T/C = 52% ($p = 10^{-3}$) or 32% ($p < 10^{-3}$), respectively. LY2603618 alone at 45 mg/kg was inactive, whereas 90 mg/kg produced T/C = 82%. The H441 tumor model showed a similar inhibitory trend. H2122 tumors were harvested at 2 h after q3dx4 of the combination or LY2603618 alone, and 26 h after PMX alone. Western analysis showed that PMX alone at 100 mg/kg induced a 2.8 fold ($p < 10^{-3}$) increase in autophosphorylation of Chk1 S296 (pChk1) over vehicle. Combined with LY2603618 at 90 mg/kg reduced this increase by 29% to 2.0 fold ($p < 10^{-3}$), whereas LY2603618 alone had no effect. H2122 cultured in RPMI 1640 with 10% dialyzed fetal bovine serum exposed for 6 days to PMX or LY2603618 alone at 100 nM inhibited cell growth by >90% ($IC_{50} = 2$ nM) or <25% ($IC_{50} = 271$ nM) respectively. Compared to vehicle, 100 nM PMX alone at 28 h induced a 14× ($p < 10^{-3}$) increase in pChk1. While LY2603618 alone had no effect, a combination involving 100 nM PMX for 21 h followed by additional 7 h with 100 nM LY2603618 induced 7.6X ($p < 10^{-3}$) increase in pChk1 over vehicle, representing a 46% reduction from PMX alone. However, pChk1 induced by PMX alone was less abated when PMX+LY2603816 were added concurrently for 28 h, or 2 h LY2603618 prior to further inclusion of PMX for 28 h total. In conclusion, compared with PMX alone, sequential treatment of PMX and LY2603618 showed enhanced antitumor activity, which could be due to interference of the PMX induced cell cycle checkpoint by LY2603618.

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Antitumor efficacy of novel hedgehog inhibitors

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Background: The Hedgehog (Hh) signaling pathway plays an important role in tissue growth and repair. Aberrant activation of Hh pathway is involved in multiple tumor types. Previous studies have demonstrated potential synergy between Hh inhibitors and *nab*-paclitaxel (Abraxane, *nab*-P) in pancreatic cancer models. Hh-Gli signaling pathway has been shown to be essential for tumor growth, recurrence, and metastasis in the HT29 human colon carcinoma model. In this study, we tested the antitumor efficacy of a series of novel Hh inhibitors alone and in combination with *nab*-P against HT29 xenografts.
Material and Methods: A series of novel Hh inhibitors were synthesized and screened in vitro for inhibition of Gli1 activity. Selected compounds were tested in male athymic mice bearing HT29 xenografts at 25, 50, 75 and 100 mg/kg by IP, qdx12 or oral dosing, bidx12. *nab*-P was intravenously administered at suboptimal dose of 10 mg/kg, q4dx3.
Results: Hh inhibitors as single agents showed modest antitumor activity. In combination, Hh inhibitors by IP or bid oral dosing significantly increased antitumor efficacy of *nab*-P. In particular, ABI-2088 consistently demonstrated superior activity and lower toxicity compared with the known Hh inhibitor GDC 0449 (Curis/Genentech) when combined with *nab*-P. ABI-2088 (100 mg/kg, bidx12, PO) + *nab*-P resulted in TGI of 91%, significantly better than *nab*-P alone (TGI 66.5%, $P < 0.001$) and GDC 0449 + *nab*-P (TGI 81.4%, $P < 0.01$).
Conclusions: A number of novel Hh inhibitors were synthesized and screened for activity in vitro and in vivo. Oral delivery of these compounds in combination with *nab*-P showed synergistic activity and promise as anticancer agents.

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Perifosine in combination with antimetabolites induces synergistic effects on cytotoxicity and apoptosis in human colon, multiple myeloma, breast, renal, and liver tumor cell lines

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Perifosine, a novel, potentially first-in-class, oral Akt inhibitor, is currently in phase III trials for advanced colorectal cancer and multiple myeloma, as well as in other phase I and phase II trials for several other tumor types. To explore novel treatment options we evaluated the synergistic potential of Perifosine in combination with selected antimetabolites in a broad panel of human cancer cell lines.
Measurement of the cellular cytotoxic/antiproliferative activity of Perifosine w/wo combination with different antimetabolites was based on the dye Resazurin, which exhibits fluorescence change relating to cellular metabolic reduction (Nociaro et al. 1993, Int. J. Oncology 3, 473). The analysis of drug combinations was conducted by using the CalcuSyn software (Biosoft, Cambridge, UK), which yielded the combination index (CI), dose reduction index (DRI) and an isobologram analysis as classification (T.C. Chou 2006, Pharmacological Reviews 58, 621–681). Perifosine and representative antimetabolites were further evaluated regarding their effects on cell cycle and induction of apoptosis.
Synergistic cytotoxic/antiproliferative activity was demonstrated for Perifosine in combination with various antimetabolites in human colon,

multiple myeloma, breast, renal, and liver tumor cell lines as indicated by combination indices below 0.7. In the colon cancer cell line SW620 cell cycle analysis revealed G2M arrest as mechanisms of action for Perifosine, whereas two representative antimetabolites, i.e. 5-Fluorouracil and 6-Thioguanine, induced S-phase arrest as expected. In combination, synergistic effects were observed in terms of apoptosis, e.g. caspase activation.
In summary, these results demonstrate potent synergistic activity of Perifosine with various antimetabolites in human colon, multiple myeloma, breast, renal, and liver tumor cell lines. Synergism seems to be based on combining G2M arrest by Perifosine and S-phase arrest by the antimetabolite resulting in synergistic induction of cellular apoptosis. Further experiments addressing Perifosine's mechanism of action in combination with antimetabolites are ongoing. Currently, Perifosine is in a phase III clinical trial in combination with Capecitabine in patients with refractory advanced colorectal cancer.

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Effects of EGFR inhibition with tyrosine kinase inhibitors on invasive properties of EGFR mutant and wild type lung cancer cells

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Background: The epidermal growth factor receptor (EGFR) pathway is known to be involved in the invasive and metastatic process. Furthermore, it is also known that activating mutations of EGFR confer increased sensitivity to small molecule tyrosine kinase inhibitors (TKI's) in the treatment of non-small cell lung cancer (NSCLC). The effects of various TKI's on lung cancer cell proliferation and survival have been previously investigated. However, the effects on invasion (and metastasis) have been studied less. Our present study investigates the effects of EGFR TKI's on the invasion of lung cancer cells with a wild-type or a mutant EGFR gene in an *in vitro* invasion assay.
Materials and Methods: The model used is based on the preparation of native collagen type I, the main interstitial matrix component of solid tumors. Three NSCLC cell lines − NCI-H358 (EGFR-WT), NCI-H1650 (EGFR-ΔE746-A750) and NCI-H1975 (EGFR-L858R/T790M) − were evaluated with several inhibitors of EGFR (erlotinib, lapatinib, BIBW2992, and cetuximab) for the effects on their invasive properties. Invasion- induced changes in cellular structure and F-actin organization were analyzed with phase contrast and confocal microscopy techniques. Invasive index, and factor shape were measured via image processing.
Results: Qualitative and quantitative analysis show that while lapatinib and cetuximab have a moderate effect on the attenuation of epidermal growth factor (EGF) stimulated invasion of mutant NCI-H1650, erlotinib and BIBW 2992 significantly abrogate cellular invasion ($P < 0.0001$). Similarly, BIBW 2992 abrogates invasion in the T790M mutant NCI-H1975 cell line ($P < 0.01$), whereas no effects are observed with any of the first-generation inhibitors. Interestingly, erlotinib significantly promoted EGF stimulated invasion of wild-type NCI-H358 ($P < 0.001$), while BIBW2992 did not.
Conclusions: These findings show that, as assessed in the pre-clinical *in vitro* collagen type I assay, erlotinib has differential effects on the invasive phenotype depending on the genomic status of the EGFR gene, promoting invasion in wild type cells. Our study also supports the use of BIBW 2992 as a therapeutic option in tumors bearing the EGFR-T790M resistance-conferring mutation.

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Distinct inhibitory properties of MEK inhibitors on pathway feedback translate into differential potency in BRAF and RAS mutant cancer cells

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Background: The RAS/RAF/MEK pathway is active in over 30% of human tumors, often due to mutation in BRAF or RAS family members. Several MEK inhibitors, aimed at treating tumors with RAS/RAF pathway alterations, are in various stages of clinical development. Despite their similarities, MEK inhibitors from distinct chemical series differ in their ability to modulate and inhibit signaling in BRAF and KRAS mutant cell lines and tumors. Here we explore the biochemical nature of this differential potency.
Results: GDC-0973 is a potent, selective, allosteric MEK1/2 inhibitor that is currently being tested in early stage clinical trials. G-573 is an allosteric MEK1/2 inhibitor from a distinct structural class with similar biochemical potency and selectivity as GDC-0973. While GDC-0973 and G-573 have similar cellular potencies in $BRAF^{V600E}$ mutant cells, G-573 displays up to 10 fold higher potency in KRAS mutant cell lines, and shows greater efficacy in vivo in KRAS mutant xenograft tumors. In vivo, GDC-0973 shows stronger maximal efficacy than G-573 in $BRAF^{V600E}$ mutant xenograft models whereas G-573 shows stronger efficacy than GDC-0973 in KRAS mutant models. To investigate the basis for the different activities of these two MEK inhibitors, we analyzed their effects on components of the RAF/MEK/ERK pathway in $BRAF^{V600E}$ vs. RAS mutant cells. We found that GDC-0973, but not G-573, increases levels of phosphorylated MEK (pMEK) and displays a potency shift in blocking pERK in KRAS vs, $BRAF^{V600E}$ mutant cells. This pMEK increase is mediated by RAF family members which are activated due to the release of negative feedback in the MAPK pathway. Although G-573 leads to a similar negative feedback release and RAF activation, it blocks MEK phosphorylation by activated RAF and is more effective at blocking downstream ERK activation in KRAS mutant cells. This effect translates into distinct cellular potencies for the two inhibitors in RAS mutant models, where the negative feedback is present, but not in $BRAF^{V600E}$ models, where it is absent.
Conclusions: These findings provide an explanation for the potency differences of MEK inhibitors in RAS vs. $BRAF^{V600E}$ mutant xenograft tumors and support a model in which potency in RAS mutant tumors correlates with the ability of MEK inhibitors to effectively block MEK activation by RAF. As a consequence, different classes of MEK inhibitors may show distinct efficacy profiles in the clinic.

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The cis/trans effect of the T790M drug resistant mutation in non-small cell lung cancer

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Background: Activating mutations of the epidermal growth factor receptor (EGFR) in non-small cell lung cancer (NSCLC) confer increased sensitivity to small molecule tyrosine kinase inhibitors (TKIs). However, despite initial response, tumors often develop the resistance conferring T790M mutation. Although resistance mechanisms of T790M have been studied, the impact of T790M arising in either *cis* or *trans* to the activated allele remains to be established. The aim of our study was to compare the effects of EGFR primary activating mutations associated with TKI sensitivity to the TKI insensitive EGFR-T790M arising in *cis* or *trans*.
Materials and Methods: The model used is based on the interleukin-3 (IL-3) dependent Ba/F3 system. We transformed the Ba/F3 cells to an epidermal growth factor (EGF) dependent system by the exogenous introduction of wild-type and mutant forms of the EGFR gene through stable transfection (wild-type EGFR, mutant EGFR and *cis* constructs) and stable co-transfection (*trans* configurations). We assessed the functionality of our constructs with two EGFR tyrosine kinase inhibitors, erlotinib and a novel irreversible inhibitor, BIBW 2992 through ^{3}[H]thymidine incorporation, MTS, Annexin V/7-AAD and western blot analysis.
Results: Our results show that T790M arising in *trans* to a primary activating EGFR mutation exhibits increased activation of AKT, ERK1/2 and STAT5 when compared to its *cis* counterpart. We also found that BIBW 2992 overcomes resistance in all erlotinib resistant T790M conformations by decreasing proliferation, increasing apoptosis and promoting G_1 cell cycle arrest.
Conclusions: The T790M mutation activates the EGFR signal transduction pathway more effectively in the *trans* than in the *cis* conformation relative to primary activating mutations. The covalent EGFR/HER2 inhibitor BIBW 2992 has activity in both conformations.

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A novel selective MET inhibitor combined with erlotinib overcomes erlotinib facilitated resistance in patient derived NSCLC xenografts in vivo

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Most advanced non-small-cell lung cancers (NSCLCs) and especially the fraction with activating epidermal growth factor receptor (EGFR) mutations initially respond to the EGFR tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib. However, most tumors develop acquired resistance to EGFR TKIs via secondary resistance mutations. The amplification of the MET oncogene is present in 20% of TKI-resistant tumors, and in half of the cases