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**The antidiabetic drug metformin - a novel therapeutic for HER2-positive breast carcinomas?**

J. Menendez[1], C. Oliveras-Ferraros[1], J. Brunet[1], A. Vazquez-Martin[1]
[1]*Fundació d' Investigació Biomèdica de Girona, Institut Català d'Oncologia de Girona, Girona, Spain*

A lower cancer-related mortality and cancer risk has been recently attributed to metformin consumption in diabetic patients. Intriguingly, the therapeutic application of metformin for Estrogen Receptor (ER)-negative breast cancer lesions in vivo appears to promote and increase the angiogenic phenotype and tumorigenic progression. Conversely, systemic treatment with metformin significantly increases life span in HER2 (erbB-2)-transgenic mice. Given our recent identification of a bidirectional linkage between endogenous fatty acid metabolism and HER2 in human breast cancer cells, we here envisioned that HER2 oncoprotein may represent a key cellular target involved in the anti-cancer actions of metformin. Metformin treatment decreased HER2 expression in a dose- and time-dependent manner (> 80% reduction) in three in vitro HER2-over-expressing breast cancer models. Metformin-induced suppression of HER2 occurred regardless the molecular mechanism contributing to HER2 overexpression (i.e. naturally by gene amplification in SKBR3 cells and ectopically driven by a viral promoter in MCF10A and MCF-7 cells stably transduced with the human HER2 cDNA), thus suggesting that metformin did not affect the transcriptional rate of the endogenous HER2 gene. Metformin treatment activated the fuel sensor AMP-activated protein kinase (AMPK). However, co-treatment with the AMPK inhibitor compound C incompletely prevented the ability of metformin to inhibit HER2. Moreover, AMPK activation upon treatment with 5-aminoimidazole-4-carboxamide (AICAR) failed to mimic metformin-induced inhibition of HER2. Specific inhibition of the AMPK pathway using small interfering RNA (siRNA) against the alpha1/2 catalytic subunits of AMPK did not prevent the anti-HER2 actions of metformin. Metformin treatment dose- and time-dependently abolished the activity of the ribosomal p70S6 kinase (p70S6K1), a downstream effector of the AMPK/mTOR pathway. Of note, ablation of endogenous p70S6K1 by siRNA was sufficient to completely protect breast cancer cells from the anti-HER2 effects of metformin. The expression status of HER2 remained insensitive to mTOR blockade by rapamycin. The ability of metformin to efficiently suppress HER2 overexpression through a direct (AMPK- and mTOR-independent) inhibition of p70S6K1 strongly suggests that the presence/absence of molecular hallmarks such as HER2 oncogene overexpression might dictate alternative breast cancer responses to the antidiabetic drug metformin.

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**Novel titanocene analogues induce apoptosis in prostate cancer epithelial cells by initiating a DNA damage response**

S. Cuffe[1], C. Dowling[1], C. Gill[1], M. Tacke[2], J.M. Fitzpatrick[1], M.P. Carty[3], R.W.G. Watson[1]
[1]*Conway Institute of Biomolecular and Biomedical Research University College Dublin, School of Medicine and Medical Science, Dublin, Ireland;* [2] *Conway Institute of Biomolecular and Biomedical Research University College Dublin, School of Chemistry and Chemical Biology, Dublin, Ireland;* [3] *National University of Ireland Galway, Department of Biochemistry, Galway, Ireland*

Background: Treatment options for locally advanced metastatic prostate cancer are extremely limited with docetaxel (Taxotere®) being the standard chemotherapy but only providing a three month survival advantage. The objectives of this study are to investigate novel titanocene analogues as possible alternative chemotherapies for advanced disease. The primary aims are to investigate the apoptotic effects of these novel titanocene analogues on prostate cells and to examine their mechanisms of action.

Materials and Methods: PwR-1E and PC-3 cell lines were grown in optimal conditions and treated with titanocene analogues at different doses and times. Apoptosis and viability were assessed by propidium iodide staining and flow cytometry and PARP cleavage. Cellular uptake and DNA binding of Titanium was measured by atomic absorption spectroscopy (Dr. JL Beltramo, Université de Bourgogne, France). Alkaline single cell gel electrophoresis was carried out using the Trevigen CometAssayTM kit to assess DNA damage and Replication Protein A (Ser 4/8) and p53 (ser 15) phosphorylation were assessed by western blotting to confirm a DNA damage response. Knock-down of p53 was achieved by si-RNA and assessed by western blotting.

Results: PwR-1E and PC-3 cells undergo apoptosis in a dose dependent manner following treatment with a range of titanocene analogues as determined by PI DNA staining and PARP cleavage. These compounds enter both cell lines and bind to DNA as confirmed by atomic absorption spectroscopy. These results confirm a correlation of increased Titanium-DNA binding and apoptotic responses. The differential apoptotic response between the PwR-1E and PC-3 cell lines correlates with the uptake of Titanium into the cells and consequently the level of DNA binding.

The titanocene compounds induce DNA damage in both cell lines as shown by the formation of 'comet tails' of DNA fragmentation upon single cell gel electrophoresis and the phosphorylation of Replication Protein A and p53. However induction of apoptosis by the titanocene compounds is not p53 dependent as demonstrated by knock-down of p53 by si-RNA in the PwR-1E cell line and no expression in the PC-3 cells.

Conclusion: These pre-clinical studies demonstrate for the first time that these novel titanocene analogues induce apoptosis in prostate cancer cell lines. Further evaluating the mechanism of action will indicate their appropriate clinical use in different stages of prostate cancer development.

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**Retinoids, in combination with Histone Deacetylase (HDAC) inhibitors, as a potential therapy for pancreatic cancer**

J. Susanto[1], E. Colvin[1], C. Scarlett[1], V. Ong[1], A. Mawson[1], M. Pinese[1], A. Biankin[1]
[1]*Garvan Institute of Medical Research, Cancer Research, Sydney, Australia*

Background: The aim of this study was to investigate the role of retinoids in combination with pharmacological agents that reverse epigenetic silencing, as potential therapies for pancreatic cancer (PC). Retinoid-based treatments, which have been used successfully in some leukaemias, have had disappointing results in other cancers. We hypothesised that this may be in part due to aberrant retinoic acid (RA) signalling. Cellular Retinoid Binding Protein 1 (CRBP1) plays a key role in RA signalling by presenting retinoids to their metabolising enzymes. In other cancers such as breast cancer, CRBP1 is frequently silenced due to epigenetic modification. Downregulation of CRBP1 expression leads to localised retinoid deficiency that could result in the downregulation of RA receptors, primarily RARβ. Restoring CRBP1 expression may restore RA signalling, enabling retinoid-based therapy to be developed in PC.

Methods: CRBP1 expression in human PC and normal samples, and PC cells lines was determined using immunohistochemistry and quantitative real time PCR (QRTPCR) respectively. Methylation status of CRBP1 promoter was investigated using methylation specific PCR. MiaPaCa2 (MP2) cells demonstrated methylation of the CRBP1 promoter and complete loss of CRBP1 expression. MP2 cells were then treated with 300μM of demethylating agent 5-aza-2'-deoxycytidine (5-AZA) and 100nM of HDAC inhibitor Trichostatin A (TSA). The expression of CRBP1 after drug treatment was measured using QRTPCR.

Results: The majority of human PC (70%) demonstrated loss or downregulation of CRBP1 expression (n=90). This was also evident in early PC precursor lesion, pancreatic intraepithelial neoplasia (PanIN), suggesting that loss of CRBP1 expression was an early event in the development of PC. Methylation of the CRBP1 promoter was identified in 28% of PC samples with loss or downregulation of CRBP1 expression (n=32), but not in normal samples (n=5). Treatment of MP2 cells with 5-AZA and TSA resulted in detectable expression of CRBP1 mRNA using QRTPCR.

Conclusion: The loss or downregulation of CRBP1 expression occurs in a significant proportion of human PC and PanIN. The loss of CRBP1 expression in the PC cell line MP2 was associated with epigenetic silencing and could be reversed pharmacologically, resulting in detectable mRNA expression. These results suggest that demethylating agents and HDAC inhibitors, in combination with retinoids, may have a therapeutic role in PC.

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**Mechanisms of EGFR-TKI induced cell death and resistance in EGFR mutant non-small cell lung cancer**

J. Ko[1], Y. Kim[1], M. Park[1], Z. Cui[1], M. Ahn[1], K. Park[1]
[1]*Samsung Medical Center, Cancer Center, Seoul, South Korea*

Although large clinical trials have shown that EGFR tyrosine kinase inhibitors (TKI) prolong survival of lung cancer patients, the precise mechanism of EGFR-TKI drug action leading to apoptotic cell death in EGFR dependent lung cancer hasn't been clearly elucidated. Moreover, patients who initially respond to TKI therapy invariably develop acquired resistance to the drugs, necessitating alternative approaches to EGFR TKI monotherapy in non-small cell lung cancer (NSCLC).

A series of in vitro and in vivo experiments were performed to better understand the cell death and resistance mechanisms induced by EGFR-TKI. In EGFR tyrosine kinase domain mutant NSCLC cells, which undergo rapid apoptosis upon treatment with EGFR-TKI, alterations of EGFR downstream signaling pathways were probed in response to TKI. Part of this cell death program is initiated by BH3 only protein, Bim. The induction of Bim and the initiation of apoptosis are mediated by a couple of EGFR downstream signaling pathways, including ERK dependent Bim phosphorylation and its inhibition. In addition to Bim induction, it was found

that other member(s) of programmed cell death participate in the completion of EGFR-TKI induced cell death in NSCLC.

In about half of the patients, the acquired resistance to EGFR-TKI monotherapy upon prolonged treatment is due to T790M secondary mutation in the kinase domain of EGFR. However, a significant portion of patients develop acquired resistance without alterations in the primary sequence of EGFR kinase domain. A few mechanisms, including receptor cross-talks and transphosphorylation by non-ErbB family members, were suggested as possible explanations of acquired EGFR independence, though the physiological relevance of these results obtained from in vitro experiments still remains unclear. As an alternative pre-clinical model system of the acquired resistance to the current reversible TKI therapy, orthotopic tumor xenografts mice were treated with EGFR-TKI for a prolonged period of time. Refractory clones obtained from the lung of such mice display a series of histological and cellular phenotypes distinct from its parental cells. A subgroup of mice developed acquired resistance through T790M mutation. Other mice, however, developed resistance independent of T790M mutations. The molecular mechanisms leading to the acquired resistance are discussed.

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**Specific inhibition of hypoxia-induced vascular endothelial growth factor expression by flavonoids in human lung cancer cells**

E. Ansó[1], M. Irigoyen[2], A. Zuazo[1], A. Rouzaut[2], J.J. Martínez-Irujo[1]
*[1]University of Navarra, Department of Biochemistry, Pamplona, Spain; [2] Centre for Applied Medical Research, School of Medicine, Pamplona, Spain*

Flavonoids are a group of polyphenolic secondary metabolites important for plant biology and human nutrition. Epidemiological studies have shown that these compounds may have an important role in explaining the favorable effects of vegetables and fruits against cancer, in special lung cancer. Previous studies have reported that some flavonoids, such as quercetin, luteolin or fisetin, induced apoptosis in several cancer cell types. Another important property related to cancer chemoprevention may be their ability to inhibit tumor angiogenesis. Cells exposed to hypoxia up regulate the expression of several transcription factors, including the hypoxia inducible transcription factor (HIF), which induces the coordinated expression of many genes involved in glucose metabolism, pH regulation and angiogenesis. Among these genes, the vascular endothelium growth factor (VEGF) plays a key role in the stimulation of tumor angio and lymphangiogenesis. We investigated the effects of a group of 40 structurally related flavonoids on the expression of VEGF and HIF-1 alpha in human lung cancer cells. We found that several of these compounds inhibited VEGF production under hypoxic conditions at non cytotoxic concentrations. We also investigated the molecular pathways involved in the inhibition of VEGF expression, including MAP kinase and PI3-kinase-signaling pathways, and the expression and transactivation of HIF-1 factor. This research leads us to analyze the structure-activity relationships for these compounds and the relevance of each pathway in the inhibition of VEGF production. Overall, it is concluded that among the wide range of biological effects that flavonoids may exert, inhibition of angiogenesis may be of great relevance in their anticarcinogenic properties.

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**The combined effect of a non selective and a selective cyclooxygenase-2 inhibitor and 5- fluorouracil treatment on HCA-7 human colorectal carcinoma cell line**

A. Réti[1], B. Budai[1], V. Komlósi[1], V. Adleff[1], G. Barna[2], A. Jeney[2], J. Kralovánszky[1]
*[1]National Institute of Oncology, Clinical Research, Budapest, Hungary; [2] Semmelweis University,1st Institute of Pathology and Experimental Cancer Research, Budapest, Hungary*

Background: 5-FU is included in many major chemotherapeutic regimens which have been statistically judged to be effective adjuvant therapy for patients with colorectal cancer. However, 5-FU itself does not substantially improve survival rates. Several NSAIDs have been tested in combination with a number of cytotoxic drugs. We hypothesized that treatment of cancer cells with 5-FU combined with indomethacin (INDO) or NS-398 might have a synergistic antiproliferative effect.

The aim of the study was to investigate whether INDO a nonselective cyclooxygenase (COX) inhibitor or NS-398, a COX-2-selective inhibitor, influence the cytotoxic effect of 5-fluorouracil on high COX-2 protein expressing HCA-7 colorectal cells. Considerable research effort was directed towards understanding the mechanism how these COX inhibitors modify the cytotoxicity of 5-FU.

Materials and methods: Sulphorhodamine B proliferation assay was used to measure the effect of 48 h 5-FU±INDO or 5-FU±NS-398 treatments on HCA-7 cells. COX-2 protein levels were analysed by Western blot and immunofluorescent method. PGE2 production was measured by ELISA. To investigate the cell cycle and apoptotic cells FACS analysis was used.

In order to understand the relative insensitivity of HCA-7 cells against 5-FU (IC50 value: 1mM) we studied the rate limiting enzyme of 5-FU catabolism dihydropyrimidine dehydrogenase (DPD)

Results: INDO or NS-398 treatment alone did not influence the proliferation of HCA-7 cells. INDO or NS-398 combinated with 5-FU significantly enhanced the proliferation inhibition caused by 5-FU alone. ($p<0.01$). The PGE2 production was reduced by 90% after 48 hours treatment with INDO or NS-398 which was similar range in case of 5-FU combinations as well. COX-2 protein levels were relatively unchanged. FACS analysis showed a delay in S phase progression and a marked decrease of G2/M fraction after treatment with 5-FU + INDO or 5-FU + NS-398.

The combined treatments also caused a significant increase in the number of apoptotic cells compared to 5-FU alone ($p<0.01$). High DPD enzyme activity was demonstrated in HCA-7 cells which was strongly reduced by both INDO and NS-398 as well.

Conclusion: 5-FU cytotoxicity against HCA-7 cells was augmented by combination with COX inhibitors which could be due at least partly to the increase of apoptotic cells and increase of the amount of 5-FU available for the anabolism as a consequence of the reduction of DPD activity.

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**NSC-mediated tumor selective therapy - towards glioma clinical trials**

K.S. Aboody[1,2], J. Najbauer[1], M.K. Danks[6], S.U. Kim[7], Metz M.Z.[1], E. Garcia[1], M.E. Barish[2], C.A. Glackin[3], B. Badie[4], J. Portnow[5]
*[1]City of Hope National Medical Center and Beckman Research Institute, Divisions of Hematology/Hematopoietic Cell Transplantation, Duarte, USA; [2]City of Hope National Medical Center and Beckman Research Institute, Neurosciences, Duarte, USA; [3]City of Hope National Medical Center and Beckman Research Institute, Molecular Medicine, Duarte, USA; [4]City of Hope National Medical Center and Beckman Research Institute, Neurosurgery, Duarte, USA; [5]City of Hope National Medical Center and Beckman Research Institute, Medical Oncology, Duarte, USA; [6]St. Jude Children's Research Hospital, Memphis, USA; [7]Division of Neurology, University of British Columbia, Vancouver, Canada*

Neural stem cells (NSCs) display inherent tumor-tropic properties that can be exploited for targeted delivery of anti-cancer agents to invasive and metastatic tumors. Malignant gliomas are the most common primary brain tumors and are considered among the deadliest of human cancers. We postulate that NSC-mediated therapy of glioma will increase tumor-selectivity and decrease systemic toxicities, and thus potentially achieve therapeutic indices sufficient to eradicate invasive and residual tumor cells that are otherwise lethal. We generated a v-myc immortalized, clonal human NSC line, HB1.F3, which has been modified to stably express the cytosine deaminase (CD) therapeutic transgene (HB1.F3.CD). CD coverts 5-fluorocytosine (5-FC) prodrug to chemotherapeutic 5-fluorouracil (5-FU). Pre-clinical safety data in mice indicate that the HB1.F3.CD NSC line is non-toxic, non-immunogenic, non-tumorigenic, and chromosomally and functionally stable over at least 15 passages. Identification of a single copy and single insertion site for both v-myc and CD genes was determined by LAM-PCR and confirmed by Q-PCR. We believe that use of this stable, sustainable, and expandable NSC line will circumvent the problems associated with characterization, senescence, and replenishment sources of primary stem cell pools. Our pre-clinical therapeutic efficacy studies using the HB1.F3.CD NSCs in combination with 5-FC prodrug in laboratory animals demonstrated 70-90% of anti-tumor responses, as measured by decreased tumor burden or increased survival time in glioma-bearing mice (and in solid tumor metastases to brain, and medulloblastoma models). Our data also indicate that HB1.F3.CD cells can be labeled with iron oxide superparamagnetic nanoparticles, which allows in vivo MRI monitoring of NSC migration to intracranial glioma in tumor-bearing mice. We now propose the use of HB1.F3.CD NSCs in human patients with recurrent high-grade glioma. The NIH Recombinant Advisory Committee has approved the use HB1.F3.CD cells for recurrent glioma at a public hearing December, 2007. We are developing a pilot study in patients with recurrent high-grade glioma to assess the safety and feasibility of HB1.F3.CD NSCs injected directly into the brain parenchyma at the time of surgical tumor resection, in combination with oral 5-FC. We postulate that HB1.F3.CD

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**Development of anti MUC1 DNA aptamers for the imaging of breast cancer**

C. Da Pieve[1], A. Perkins[2], S. Missailidis[1]
*[1]The Open University, Chemistry, Milton Keynes, United Kingdom; [2] University of Nottingham, Medical Physics Medical School, Nottingham, United Kingdom*