ANTICANCER RESEARCH *24*: 505-538 (2004)

ABSTRACTS OF THE INTERNATIONAL CONFERENCE

"BIOTECHNOLOGY, CANCER AND DRUG RESISTANCE: NEW TARGETS, NEW DIAGNOSIS AND NEW TREATMENT FOR TUMOURS RESISTANT TO CURRENT THERAPIES"

7-8 July, 2003
Clontarf Castle, Dublin, Ireland

Abstracts of Oral Presentations

NEW INSIGHTS INTO THE CHRONIC LYMPHOPROLIFERATIVE DISORDERS

Denis Alexander

Department of Haematology, Belfast City Hospital and School of Biomedical Sciences, University of Ulster at Coleraine, N. Ireland. E-mail: Denis.Alexander@bll.n-i.nhs.uk

The chronic lymphoproliferative disorders (CLPD), including non Hodgkin's lymphoma (NHL) and chronic lymphocytic leukaemia (B-CLL), account for approximately 45% of haematological malignancies. There are geographical variations in the distribution of CLPD, but B-CLL is the most common leukaemia in Europe and N America.

Understanding the biology of disease in CLPD, particularly B-CLL, has lagged behind that of other haematological malignancies. Recent research has gone some way towards answering three major questions, namely:

What is the nature of the recurring acquired cytogenetic abnormalities in B-CLL and how do these contribute to its biology?

How is the conundrum of malignant B-CLL cells, which won't die *in vivo* but don't survive in *in vitro* culture systems, explained?

Is the T cell expansion frequently seen in B-CLL a true anti-tumour response?

Most attempts to culture B-CLL *in vitro* fail, leading to difficulties in applying conventional karyotyping techniques. Thus, acquired cytogenetic abnormalities went undetected until recently, when the application of interphase fluorescent *in situ* hybridisation (FISH) and polymerase chain reaction (PCR) based molecular techniques showed a range of recurring cytogenetic abnormalities with highly predictive prognostic capabilities. This has helped to explain why, for example, one third of B-CLL patients presenting with good prognosis early clinical stage will in fact have progressive disease. Early indications are that, in B-CLL, unmutated IgV_H genes, 17p mutations or deletions and 11q23 abnormalities are particularly associated with poor outcomes. Although these features have obvious clinical and therapeutic implications, they are studied infrequently and few attempts are made at risk stratification of patients at presentation.

Whilst most B-CLL cells are thought to be arrested in the G0/G1-phase of the cell cycle, recent evidence has shown that proliferation centres of large nucleolated CLL cells are present in most lymph nodes and that there may be an unexpectedly high turnover of B-CLL cells *in vivo*. It is now recognised that the microenvironment is essential in B-CLL and bystander non-humoral cells play an indispensable role in the survival and accumulation of leukaemic cells. This has led to the concept of 'nurse cells' and attempts to identify candidates. T_{helper} cells are distributed throughout these proliferation centres and crosstalk contributes to survival abnormalities and defective apoptosis. T_{helper} cells produce large quantities of cytokines, some of which can up-regulate bcl-2. Survivin integrates apoptosis and cell cycle and controls apoptosis by blocking caspases. T cells are important for the action of survivin and they may gather in proliferation centres through attraction by chemokines produced by B-CLL cells.

The expansion in circulating CD4+ and, in particular, CD8+ T cells in B-CLL has been recognised for some time. This has been proposed to be an anti-tumour response. Recent evidence, however, suggests that these expanded T cell populations result from reactivation of cytomegalovirus (CMV) in immunocompromised individuals and may in fact be a poor prognosis indicator. CMV infects the majority of the population, is usually asymptomatic and is contained by a sustained immune response. Serological evidence of reactivation is accompanied by an expansion of the CD28-/CD57+ subset of CD4+ T cells and CD45RA+/CD27-CD8+ T cells, both of which are CMV-specific, and is associated with decreased survival in the normal elderly population. CMV sero-positivity may have clinical significance in B-CLL. The possibility of an anti-tumour immune response remains unproven.

UBC9 AND DAXX PARTICIPATE IN RESPONSE OF TUMOR CELLS TO TOPOISOMERASE I INHIBITORS AND OTHER ANTICANCER DRUGS

Yin-Yuan Mo, Yanni Yu, P. L. Rachel Ee and William T. Beck

Departments of Biopharmaceutical Sciences and Molecular Genetics, University of Illinois at Chicago, Chicago, IL 60612, U.S.A. E-mail: wtbeck@uic.edu

Aims: Ubc9 is an E2 conjugating enzyme required for sumoylation, which has been implicated in regulating the subcellular distribution of many proteins, including RanGAP1, PML, p53, c-Jun, IKB, topoisomerase (topo) II and topo I (Müller *et al*, Nature Reviews Molec Cell Biol *2*:202-210, 2002). We have shown previously that Ubc9 is important for sumoylation and nucleolar delocalization of topo I in response to topo I inhibitors such as topotecan (Mo *et al*, J Biol Chem *277*:2958-2964, 2002). However, the role of Ubc9 in tumor drug responsiveness is not clear. In this study, we overexpressed a Ubc9 dominant negative mutant (Ubc9-DN)

in MCF7 cells and asked what happens to topo I and other proteins. *Results:* While Ubc9-DN reduced topo I activity, cytotoxicity assays indicated that Ubc9-DN unexpectedly sensitized the cells to topotecan as well as other anticancer agents such as VM-26 and cisplatin. To understand possible mechanisms of Ubc9-mediated drug responsiveness, we examined several apoptosis-related proteins, one of which is Daxx, a Fas-associated protein. Daxx plays a role in Fas-mediated apoptosis by participating in a caspase-independent pathway through activation of Ask1 and JNK and has been shown to physically interact with Ubc9. We also detected the interaction of Ubc9 with Daxx in several cell lines. Intriguingly, we found that cells expressing Ubc9-DN accumulate more cytosolic Daxx than control cells. Although Daxx resides in both the cytoplasm and nucleus, cytosolic Daxx is believed to play a role in cell apoptosis. The interaction of Ubc9 with Daxx and subsequent alteration in the subcellular localization of Daxx may contribute to the observed increased sensitivity to the anticancer drugs in cells expressing Ubc9-DN. Finally, we found that overexpression of Daxx increased the sensitivity of cells to anticancer drugs, while knockdown of Daxx by siRNA decreased the cells' sensitivity to the same drugs. *Conclusion:* Together, these results suggest roles for both Ubc9 and Daxx in tumor drug responsiveness.

OVERCOMING MULTIDRUG RESISTANCE WITH NON-CYTOTOXIC TAXANES

Tracy A. Brooks, Hans Minderman, Kieran L, O'Loughlin, Paula Pera, Iwao Ojima, Maria R. Baer and Ralph J. Bernacki

Roswell Park Cancer Institute, Elm and Calton Streets, Buffalo, NY 14263, U.S.A. E-mail:Tracy.Brooks@RoswellPark.org

Overexpression of ATP-binding cassette (ABC) transport proteins, including P-glycoprotein (Pgp), multidrug resistance protein (MRP-1) and breast cancer resistance protein (BCRP), is a well-characterized mechanism of multidrug resistance (MDR). The cytotoxic taxanes paclitaxel and docetaxel stabilize microtubules, causing cellular arrest during mitosis and apoptosis. These agents are substrates for Pgp-mediated efflux, whereas the semi-synthetic taxane analog, ortataxel, by inhibiting Pgp-mediated transport, is not. We have recently demonstrated that ortataxel also modulates drug efflux mediated by MRP-1 and BCRP. Because the cytotoxic properties of ortataxel are less favorable for development as an MDR modulator, we sought to identify non-cytotoxic, taxane-based, broad-spectrum modulators. A series of non-cytotoxic taxane-based reversal agents (tRA's) were designed as Pgp modulators by eliminating the C-13 side-chain important for microtubule stabilization. Twenty tRA's were selected for study based on their activity in Pgp modulation, evidenced by a decrease in the IC_{50} of paclitaxel in Pgp-overexpressing MDA435/LCC6^{mdr1} cells. Effects of the tRA's on mitoxantrone (a Pgp, MRP-1 and BCRP substrate) cytotoxicity and retention in myeloma cell lines 8226/Dox6 (Pgp) and 8226/MR20 (BCRP), and human myeloid leukemia cell line HL60/Adr (MRP-1) were evaluated by Coulter counter, wst-1 (water soluble reagent similar to MTT) colorimetric viability assay and flow cytometry, where the Kolmogorov-Smirnov statistic, denoted as a D-value, was used to compare histograms. Further cytotoxicity studies examining the effects of the lead tRA on classical anthracyclines were carried out in the breast cancer cell lines MCF7/S, MCF7/R (Pgp), MCF7/MRP1-10 (MRP-1) and MCF7/AdrVp3000 (mutated BCRP) with the SRB colorimetric assay. All experiments were done in a minimum of triplicate, and the mean and standard error data are reported. Of the screening of twenty tRA's, six modulated MRP-1 and ten modulated BCRP, in addition to modulating Pgp. Four tRA's - 98006, 98007, 99018 and 99020 - strongly modulated efflux and significantly enhanced cytotoxicity of mitoxantrone in each of the MDR cell lines in a dose-dependent manner, lowering the IC_{50} by 31% - 97%. tRA 98006 was identified as the lead tRA for broad-spectrum modulation based on the findings from the myeloma and myeloid leukemia cell lines, and was further examined in the breast cancer cell line panel. The IC_{50}s of the classical anthracyclines doxorubicin, daunorubicin and mitoxantrone (all subject to efflux by Pgp, MRP-1 and BCRP) were lowered by up to 95%. tRA 98006 is able to modulate Pgp-, MRP-1- and BCRP-mediated efflux of a variety of agents in multiple cell line systems, proving to be a broad-spectrum modulator with potential for clinical applications.

RIBOZYMES AND SiRNA FOR SPECIFIC mRNA DOWN-REGULATION

Carmel Daly, Deirdre Cronin, Helena Joyce, Yizheng Liang and Martin Clynes

National Institute for Cellular Biotechnology, Dublin City University, Dublin 9, Ireland. E-mail: carmel.daly@dcu.ie

In order to assign functions to genes identified as having altered expression in processes such as drug resistance, apoptosis resistance or metastasis, it is necessary to modify the genes expression in cell systems. This is carried out by gain of function studies (cDNA transfection) and loss of function studies (ribozyme, RNAi or gene knock-out). Ribozymes are RNA molecules that contain stretches of nucleotides that base-pair with complementary RNA in their target and have a catalytic section that cleaves the bound RNA. In a human lung cell line *mdr1* and caspase 3 RNA

were down-regulated by hammer-head ribozymes. In the same human lung cell system caspase 3 was also targeted by RNAi. RNAi is the mechanism by which double-stranded RNA (dsRNA) specifically suppresses the expression of a gene bearing its complementary sequence. Assays to detect down-regulation of mRNA and protein as well as functional assays were carried out on each set of transfections.

POLYUNSATURATED FATTY ACIDS: MOLECULAR AND CELLULAR BASIS FOR ANTICANCER ACTION

Rosaleen Devery

National Institute for Cellular Biotechnology, Dublin City University, Dublin 9, Ireland. E-mail: rosaleen.devery@dcu.ie

Lipids are promising molecules in cancer prevention and the potentiation of cancer treatment. Certain polyunsaturated fatty acids including their metabolites are known to exert anticancer functions, such as induction of lipid peroxidation, apoptosis, growth arrest, reduction in metastatic cascade, reversal of drug resistance and enhancement of chemosensitivity and radiotherapy. The vulnerability of cancer cells to certain essential polyunsaturated fatty acids has been attributed to an impaired fatty acid desaturation enzyme system by which fatty acid and metabolite profiles are altered in cancer cells. A number of studies in the past decade have singled out particular fatty acids, geometrical and positional isomers of linoleic acid, *i.e.*, conjugated linoleic acids (CLA) as potent inhibitors of tumorigenesis in animal models of chemically-induced cancer. Potential mechanisms of action have been identified which include reduction of cell proliferation and angiogenesis, alterations in the components of the cell cycle, induction of apoptosis, regulation of gene and oncogene expression by affecting the activity of transcription factors and modulation of lipid metabolism *via* the involvement of the peroxisome proliferator activated receptors (PPARs). The majority of research to date has been conducted using synthetic mixtures of CLA isomers. The *c*9, *t*11-CLA and the *t*10, *c*12 -CLA isomers predominate in these mixtures. In the last few years, research with individual CLA isomers has focused exclusively on these two isomers. The reason for this is that highly purified preparations of these two CLA isomers have been successfully produced, while the other isomers have yet to be acquired in sufficient quantities. Little has been done to determine the activity and mechanisms of isomers other than these two, nor of any of their metabolites. Research carried out in our laboratory showed that the *c*9, *t*11 -CLA isomer (at 5 μg/ml) caused a 50% reduction in viability of the MCF-7 human breast cancer cell line following 4 days of incubation and was similar to the CLA mixture of isomers (Miller *et al*, Lipids 36:1161-1168, 2001). The t10,c12 -CLA isomer at 16 μg/ml reduced viability by 50%. By contrast, linoleic acid (at 5 and 16 μg/ml) had a negligible effect on growth of MCF-7 cells. We have reported similar growth inhibition by CLA isomers on two human colon cancer cell lines and have identified induction of lipid peroxidation, modulation of arachidonic acid distribution and eicosanoid formation and down-regulation of specific antiapoptotic proteins by CLA isomers as potential mechanisms of action of CLA. Given the importance of arachidonic acid metabolism to lipid signalling pathways and apoptosis, apoptosis-inducing lipids could conceivably have an important role in adjunct anticancer therapy. Based on current understanding of CLA and other essential polyunsaturated fatty acids, the issues and barriers confronting the use of CLA as an adjunct therapy to chemotherapeutic agents will be discussed. This exercise should help us in identifying the gaps of knowledge that need to be addressed in the near future.

ROLE OF PROTEASES IN CANCER INVASION AND METASTASIS.

Michael J. Duffy

Dept. of Nuclear Medicine, St Vincent's University Hospital and Conway Institute of Biomolecular and Biomedical Science, University College Dublin, Dublin, Ireland. E-mail: Michael.j.duffy@ucd.ie

It is the ability to invade and metastasise that ultimately determines prognosis in patients with cancer. One of the key groups of molecules involved in invasion and metastasis are the proteases such as urokinase plasminogen activator (uPA), cathepsins such as cathepsin B, D and L and various metalloproteases (MMPs). These proteases catalyse degradation of the extracellular membrane, promote angiogenesis and stimulate cellular migration, allowing cancer cells to invade locally and metastasise to distant sites. If specific proteases are directly and causally involved in cancer spread, they have the potential to be markers of metastatic potential or prognosis in cancer. One of the striking examples of a relationship between high levels of a protease and poor prognosis is uPA in breast cancer. In breast cancer, the prognostic impact of uPA was found to be both independent of traditionally used factors and prognostic in patients with axillary node negative disease. The prognostic value of uPA in axillary node-negative breast cancer patients was recently confirmed in both a prospective randomised trial and a pooled analysis, *i.e.*, 2 different level 1 evidence (LOE-1) studies.

Conclusion: uPA is amongst the first biological prognostic factors to have its clinical value validated using LOE-1

evidence studies. Determination of uPA may therefore help identify low risk node-negative breast cancer patients for whom adjuvant chemotherapy may be unnecessary.

DNA MICROARRAY ANALYSIS OF CANCER

Geraldine Grant

Department of Molecular and Microbiology,
George Mason University, Manassas VA 20110, U.S.A.
E-mail: ggrant1@gmu.edu

Microarray technology has presented the scientific community with a compelling approach that allows for simultaneous evaluation of all cellular processes at once. Cancer being one of the most challenging diseases due to its polygenic nature presents itself as a perfect candidate for evaluation by this approach. Several recent articles have provided significant insight into the strengths and limitations of microarrays. Nevertheless, there are strong indications that this approach will provide new molecular markers that could be used in diagnosis and prognosis of cancers (1,2). To achieve these goals it is essential that there is a seamless integration of clinical and molecular biological data that allows us to elucidate genes and pathways involved in various cancers.

To this effect we are currently evaluating gene expression profiles in human brain, ovarian, breast and hematopoietic, lung, colo-rectal, head and neck, and biliary tract cancers. To address the issues we have a joint team of scientists, doctors and computer scientists from two Virginia universities and a major healthcare provider. The study has been divided into several focus groups that include; Tissue Bank Clinical & Pathology Laboratory Data, Chip Fabrication, QA/QC, Tissue Devitalizations, Database Design, and Data Analysis, using multiple microarray platforms. Currently over 300 consented patients have been enrolled in the study with the largest number being that of breast cancer patients. Clinical data on each patient is being compiled into a secure and interactive relational database and integration of these data elements will be accomplished by a common programming interface. This clinical database contains several key parameters on each patient including, demographic (risk factors, nutrition, co-morbidity, familial history) histopathology (non genetic predictors), tumor, treatment and follow-up information. Gene expression data derived from the tissue samples will be linked to this database, which allow us to query the data at multiple levels.

The challenge of tissue acquisition and processing is of paramount importance to the success of this venture. A tissue devitalization timeline protocol was devised to ensure sample and RNA integrity. Stringent protocols are being employed to ascertain accurate tumor homogeneity, by serial dissection of each tumor sample at 10μM frozen sections followed by histopathological evaluation. The multiple platforms being utilized in this study include Affimetrix™, Oligo-Chips and custom-designed cDNA arrays. Selected RNA sample will be evaluated on each platform between the groups. Analysis steps will involve normalization and standardization of gene expression data followed by hierarchical clustering to determine co-regulation profiles.

The aim of this conjoint effort is to elucidate pathways and genes involved in various cancers, resistance mechanisms, molecular markers for diagnosis and prognosis.

References

1 van't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT, Schreiber GJ, Kerkhoven RM, Roberts C, Linsley PS, Bernards R and Friend SH: Nature *415*: 530-536, 2002.

2 Bertucci, F, Viens P, Tagett R, Nguyen C, Houlgatte R and Birnbaum D: Lab Invest *83*: 305-316, 2003.

THERAPEUTIC POTENTIAL OF VEGF (VASCULAR ENDOTHELIAL GROWTH FACTOR) ANTAGONISTS

Judith Harmey

Dept. of Surgery, RCSI Education & Research Centre, Beaumont Hospital, Dublin 9, Ireland.
E-mail: jharmey@rcsi.ie

Angiogenesis is critical for both tumour growth and metastasis. Vascular endothelial growth factor (VEGF) was initially identified as an endothelial cell-specific mitogen and is a potent pro-angiogenic cytokine. We have previously shown that VEGF acts as a tumour cell survival factor. Antibodies raised against VEGF have been shown to suppress tumour growth *in vivo*, indicating that VEGF antagonists have therapeutic implications as inhibitors of tumour-induced angiogenesis. To date, a number of VEGF receptors have been identified, namely Flt-1, Flk-1/KDR and more recently, Neuropilin-1. In this study, we aimed to identify VEGF receptors on breast tumour cells and develop receptor-specific blocking peptides.

Total RNA was isolated from murine (4T1) and human (MDA-MB-231) mammary adenocarcinoma cells of metastatic origin. cDNA was synthesised by reverse transcriptase for PCR using primers specific for the VEGF receptors Flt-1, Flk-1/KDR and Neuropilin-1. Human umbilical vein endothelial cells (HUVEC) were used as a

positive control. Neuropilin-1 expression was also examined by Western blot. Peptides were designed to block Neuropilin-1 (22 mer) and KDR (7 mer) tumour cell VEGF receptors. Cells were cultured in 12-well plates for 24 hours following treatment with Neuropilin-1 (125μg/ml) and KDR (250μM) blocking peptides. Tumour cell apoptosis was assessed based on annexin-V binding to phosphatidylserine expressed on the surface of apoptotic cells or caspase activation. Flow cytometry was performed using FACscan analysis. Peptide binding to cells was assessed using confocal microscopy.

Flt-1 and Flk-1/KDR receptor expression were detected in 4T1 and MDA-MD-231 tumour cells by RT-PCR. In addition, Neuropilin-1 was identified on both tumour cell lines. The Neuropilin-1 blocking peptide induced significant tumour cell apoptosis in 4T1, MDA-MB-231 and HUVEC cells relative to VEGF control peptides at a similar concentration ($p<0.05$). Blockade of the VEGF receptor, KDR, induced apoptosis in endothelial cells but not in 4T1 and MDA-MB-231 breast tumour cells.

Blocking VEGF receptors induces both endothelial cell and tumour cell apoptosis. Further studies are warranted to determine the inhibitory effects of receptor-blocking peptides on tumour cell apoptosis *in vivo*.

IMPLICATIONS OF GENOMIC PROFILING FOR THE FUTURE DEVELOPMENT AND USE OF 5-FLUOROURACIL

Patrick G. Johnston

Department of Oncology, Cancer Research Centre, Queen's University Belfast, Belfast, N. Ireland. E-mail: p.johnston@qub.ac.uk

The fluoropyrimidine 5-Fluorouracil (5-FU) is widely used in the treatment of a range of cancers including colorectal, breast and cancers of the aerodigestive tract. Fluoropyrimidines were developed in the 1950s following the observation that rat hepatomas utilized uracil more rapidly than normal tissues, suggesting that uracil metabolism was a potential target for antimetabolite chemotherapy. The mechanism of 5-FU cytotoxicity of has been ascribed to the misincorporation of fluoronucleotides into RNA and DNA and to the inhibition of the nucleotide synthetic enzyme thymidylate synthase (TS). While 5-FU in combination with other chemotherapeutic agents improves response rates and survival in breast and head and neck cancers, it is in colorectal cancer that 5-FU has had the greatest impact. It has been demonstrated that 5-FU-based chemotherapy improves overall and disease-free survival of patients with resected stage III colorectal cancer. Nonetheless, in the metastatic disease setting, response rates for 5-FU-based chemotherapy as a first-line treatment for advanced colorectal cancer are only 10-20%. Combination of 5-FU with the newer chemotherapies irinotecan (CPT-11) and oxaliplatin has improved the response rates of advanced colorectal cancer to 40-50%. However, despite these improvements, new therapeutic strategies are urgently needed. DNA microarray technology has the potential to identify novel genes that may play key roles in mediating resistance to 5-FU-based chemotherapy. Such genes may be therapeutically valuable as predictive biomarkers of 5-FU chemosensitivity and/or provide new molecular targets that overcome drug resistance. This talk will review how pre-clinical studies have impacted on the clinical use of 5-FU and discusses how DNA microarray profiling may affect its future clinical application.

METALLOPROTEASES AND THEIR ROLE IN TUMOUR INVASION AND METASTASIS

Susan McDonnell

School of Biotechnology, National Institute for Cellular Biotechnology, Dublin City University, Dublin 9, Ireland. E-mail: Susan.McDonnell@dcu.ie

Although there have been tremendous improvements in the diagnosis and treatment of cancer over the past decade, many cancer patients still die because their tumour has metastasised or spread to other sites in the body. A full understanding of the processes that lead to metastasis will be crucial if we are to be able to effectively treat cancer. Recent studies of metastasis have revealed it as an arduous process requiring many steps which we can now begin to explore at the cellular and molecular level. First, cells must detach from the primary tumour and move into the blood vessels that nourish it. They are then carried in the general circulation until they lodge in a capillary bed and penetrate the blood vessel cell wall, invading the surrounding tissues and establishing a new tumour mass. As can be seen from this scenario, tumour cells must manifest a variety of properties in order to metastasise. In the past decade numerous studies have identified many of the players involved in this process including members of the matrix metalloprotease (MMP) family of enzymes and cell adhesion molecules like integrins and CD44. The MMPs are a family of highly conserved zinc-dependent endopeptidases which collectively are capable of degrading components of the extracellular matrix (ECM). They have been shown, through numerous *in vitro* and *in vivo* studies, to be key role players in both normal and disease processes. However, despite striking activity in pre-clinical models, the clinical development of the matrix metalloproteinase inhibitors

(MMPIs) has been difficult and the results of phase III trials have been disappointing. Despite the problems encountered, it is hoped that the MMPIs may yet provide another mechanism for the long-term control of metastatic disease. Furthermore, important lessons can be learnt from their development that are relevant to the development of other biological agents. However, these genes are only partly responsible for the metastatic behaviour of cells and many outstanding questions remain to be answered concerning the changes in gene expression that are required for metastasis. Understanding the gene expression patterns responsible for invasion and metastasis of tumour cells will have a profound influence on the diagnosis and therapy of metastatic disease. We have established an *in vitro* colorectal cell model using non-metastatic and metastatic cell lines established from the same patient. We have also transfected these cell lines with cDNAs encoding the genes for MMP-7 and MMP-9. Using cDNA microarray technology we aim to identify the gene expression profiles relevant for tumour invasion and metastasis. The results of these experiments will help clarify the molecular mechanisms involved in metastasis and could lead to the development of molecular targets for innovative anti-cancer therapies.

DRUG EFFLUX PUMPS IN FUNGI: THE INS AND OUTS OF ANTIFUNGAL AGENTS

Gary Moran

Microbiology Research Unit, School of Dental Science, University of Dublin, Trinity College, Dublin 2, Ireland. E-mail: Gary.moran@tcd.ie.

Multidrug resistance in tumour cells is often caused by the increased efflux of a wide variety of drugs, mediated by P-glycoprotein, a member of the superfamily of ATP binding cassette (ABC) transporters. Members of this gene family have also been identified in eukaryotic microorganisms and have been implicated in antimicrobial drug resistance. In pathogenic fungi, especially yeasts of the genus *Candida*, multidrug resistance is increasingly common and recent studies have implicated efflux pumps of the ABC transporter and major facilitator superfamilies (MFS) in clinical resistance to antifungal drugs. We have identified two multidrug transporter encoding genes in *Candida dubliniensis*, a recently described species of yeast responsible for opportunistic infections in immunocompromised patients. These two genes, *CDR1* (*C*andida *D*rug *R*esistance, encoding an ABC transporter) and *MDR1* (encoding a protein of the MFS), have been shown by heterologous gene expression in *Saccharomyces cerevisiae* and targeted gene deletion to mediate resistance to a wide variety of metabolic inhibitors and clinically useful antifungal drugs. Overexpression of these genes has been implicated in the resistance of *C. dubliniensis* clinical isolates to the antifungal drug fluconazole, the primary antifungal used in the treatment of oral candidosis. In studies where *C. dubliniensis* was exposed to fluconazole *in vitro*, drug resistant derivatives could be selected from previously susceptible populations, due to the selection of mutations which lead to increases in expression of either the CDR1 or MDR1 genes. Unlike prokaryotic microrganisms, horizontal gene transfer does not seem to play any role in the acquisition of drug resistance in pathogenic fungi, thus highlighting a further similarity to the acquisition of drug resistance in tumour cells. Recent genome sequencing projects in fungi have revealed the existence of large families of MFS and ABC transporters. Future studies will concentrate on characterising the substrate specificities of these proteins and elucidating the transcriptional networks that control their expression.

GENOMIC IMPRINTING AND CANCER

Catherine M. Nolan

Zoology Department, University College Dublin, Dublin 4, Ireland. E-mail: Catherine.Nolan@ucd.ie

Only a small number of genes in the mammalian genome are known to be imprinted. However, these genes are involved in processes such as intercellular signaling, RNA processing and cell cycle control, and have important effects on normal growth and behavior. Deregulation of imprinted gene expression has been implicated in numerous pathological conditions in humans and contributes to the development of cancer. We are interested in the potential role of a multifunctional receptor, known as the mannose 6-phosphate receptor and also as the IGF-II receptor (M6PR/IGF-IIR), in tumor suppression. The gene coding for this receptor is somewhat unusual: it is imprinted in rodents and artiodactyls (cows, sheep and pigs) and expressed from the maternally-inherited allele, but is apparently not imprinted in humans, where it is biallelically expressed. The biological activities of the receptor (targeting IGF-II for degradation, promoting activation of latent TGF-β, targeting acid hydrolases to lysosomes) suggested that the receptor might function in tumor suppression: observations in human tumors support this idea. However mice that inherit an inactivated *M6P/IGF-IIR* allele from their mother die at birth, and this complicates analysis of the receptor function in post-natal life. We are addressing this problem using mice that are deficient in receptor expression in a tissue-specific manner, and by studying receptor function in non-rodent animal models.

DRUG TRANSPORT AND MULTIDRUG RESISTANCE IN CANCER

Robert O'Connor

National Institute for Cellular Biotechnology, Dublin City University, Dublin 9, Ireland. E-mail: Robert.oconnor@dcu.ie

Multiple drug resistance (MDR) is a significant cause of treatment failure in cancer. Cellular pumps belonging to the ABC transporter family of proteins are thought to be an extremely important causative factor of MDR in many cancers treated with conventional cytotoxic therapy. Pump proteins including P-gp (MDR-1) and MRP-1 can actively efflux a number of the most broadly active chemotherapy drugs from cancer cells by utilising energy derived from the de-phosphorylation of ATP. Clinically, these efflux mechanisms have been a tantalising target for therapeutic intervention for several years but trials to date have been largely disappointing.

Several years ago our research group discovered that a small number of agents from the NSAID class of therapeutic drugs could synergise the toxicity of several important cancer drugs. This effect was not related to their NSAID action but was shown to be due to inhibition of MRP-1 -mediated drug efflux. We have examined the therapeutic potential of this inhibition and this has ultimately led to an ongoing clinical trial of one agent, sulindac, as a potential MRP-1 resistance circumvention agent. Several models of resistance have been explored and we have shown that the effect is due to the inhibitor acting directly on drug uptake and efflux in cancer cells. Interestingly, the research has also shown that there is significant variation in the background uptake and efflux of cancer drugs in cancer cells; an observation which we are now attempting to characterise further.

MOLECULAR MARKERS FOR CLASSIFICATION OF THERAPEUTIC RESPONSE AND OUTCOME IN BREAST CANCER

Lorraine O'Driscoll

National Institute for Cellular Biotechnology, Dublin City University, Dublin 9, Ireland. E-mail: lorraine.odriscoll@dcu.ie

Introduction: Breast cancer is now a leading cause of female cancer death in the western world, yet the cellular mechanisms responsible for its occurrence and progression, and its development of multi-drug resistance are poorly understood. *Aim*: The aim of this retrospective study was to investigate the expression and prognostic relevance of a range of apoptosis- and multi-drug resistance- related genes in a large number of invasive breast tumour biopsies. *Methods*: Using RT-PCR and immunohistochemical techniques, the expression of survivin, survivin-ΔEx3, survivin-2B, mcl-1, bcl-2, bag-1, bax-α, galectin-3, MDR-1 and MRP-1 mRNAs and, as appropriate, their corresponding proteins were analysed. Statistical (univariate and multivariate) analyses of the results were performed using SPSS 10.1 software package. Descriptive statistics were used to summarise patient characteristics and statistical analysis of the results was performed using Pearson's X^2 test to investigate relationships between mRNA expression, protein expression and clinicopathological and histopathological findings. Kaplan-Meier survival curves were established and were subsequently checked using the log-rank, Breslow and Tarone-ware tests to assess the prognostic significance of expression of analysed mRNAs and proteins in tumour cells. Multivariate survival analyses were performed using the Cox regression backward stepwise likelihood ratio. *Results*: Overall, survivin, survivin-ΔEx3, survivin-2B, bcl-2, bag-1, galectin-3, bax-α and MRP-1 mRNAs were detected in 68%, 54.7%, 9.4%, 78.4%, 80.9%, 98.9%, 97.8% and 72.8%, respectively, of tumour specimens (1). Uniquely among the mRNAs analysed here, the expression of bcl-2 correlated significantly with disease outcome, with bcl-2 expression indicative of favourable outcome in terms of both relapse-free survival (RFS) and overall survival (OS). Protein analysis also indicated bcl-2 expression to be favourably associated with outcome for patients. These results indicate that the group studied was a representative breast cancer population. Overall, 60% of tumours were positive for survivin protein and, although survivin expression was independent of patients' age, tumour size and grade, nodal and estrogen receptor status, its nuclear expression (in >20% of tumour cells) was found to be a significant prognostic indicator of favourable outcome, in terms of RFS ($p<0.001$) and OS ($p<0.01$) (2). Although no prognostic relevance was associated with MRP-1 protein expression in the total population studied, analysis of sub-populations indicated an unfavourable association between MRP-1 expression in >25% of tumour cells and both RFS ($p=0.0181$) and OS ($p=0.0171$) for patients who had received chemotherapy and had grade III tumours. Whereas MDR-1 p-glycoprotein (p-gp) expression was strongly associated with advanced histological grade, no significance association was found between expression of this protein and any of the other clinicopathological characteristic analysed; p-gp was not found to be a prognostic factor. Similarly, mcl-1 protein expression did not correlate with tumour size, grade, lymph node or ER status, age of patient at diagnosis, or disease outcome. *Conclusion*: This study indicates survivin, MRP-1 and bcl-2 protein expression to

be independently associated with both RFS and OS in invasive breast cancer and supports future functional studies targeted at manipulation of their expression to improve outcome for breast cancer patients.

References

1 O'Driscoll L, Linehan R, Kennedy SM, Cronin D, Purcell R, Glynn S, McDermott EW, Hill AD, O'Higgins NJ, Parkinson M and Clynes M. Lack of prognostic significance of survivin, survivin-ΔEx3, survivin-2B, galectin-3, bag-1, bax-α and MRP-1 mRNAs in breast cancer. Cancer Lett *201*: 225-36, 2003.

2 Kennedy SM, O'Driscoll L, Purcell R, Fitz-Simons N, McDermott EW, Hill AD, O'Higgins NJ, Parkinson M, Linehan R and Clynes M. Prognostic importance of survivin in breast cancer. Br J Cancer *88*: 1077-1083, 2003.

Acknowledgements

This work was supported by grants from The Health Research Board of Ireland, Enterprise Ireland, Cancer Research Ireland, The Irish Higher Education Authority's PRTLI program and Research Foundation, Royal Victoria Eye and Ear Hospital, Ireland.

ABC TRANSPORTERS AND DRUG RESISTANCE

Balazs Sarkadi[1], Zs. Szentpιtery[1,2], E. Bakos[2] and A. Vαradi[2]

[1]National Medical Center, Institute of Haematology and Immunology, Membrane Research Group of the Hungarian Academy of Sciences, Budapest, Hungary; [2]Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, Budapest, Hungary. E-mail: sarkadi@biomembrane.hu

A major form of resistance against a variety of the currently used antineoplastic agents involves the function of membrane proteins that extrude cytotoxic molecules, thus keeping intracellular drug concentration below a cell-killing threshold. These so called multidrug transporters belong to the superfamily of ATP Binding Cassette (ABC) proteins, present from bacteria to men. The medical significance of multidrug resistance (MDR) ABC proteins exceeds their role in cancer therapy resistance, as their transport activity has an important effect in general pharmacology, that is in modulating the absorption, distribution and excretion of numerous pharmacological compounds.

The P-glycoprotein (MDR1)-mediated multidrug resistance was the first discovered and probably still is the most widely observed mechanism in clinical multidrug resistance. However, other efflux pumps also play a significant role in transport-associated drug resistance. There are two other ABC transporters which have been definitely demonstrated to participate in the multidrug resistance of tumors: the multidrug resistance protein 1 (MRP1), and the mitoxantrone resistance protein (MXR/ABCG2) In addition, overexpression of MRP2, MRP4 and MRP5 may also cause certain forms of drug resistance.

Different tumors with MDR-ABC protein overexpression often show primary resistance to cancer chemotherapy. In addition, cancer chemotherapy itself might induce the overexpression of these proteins, so that the multidrug resistant clones become less sensitive to chemotherapy (secondary drug resistance). Treatment failure due to multidrug resistance is also found in connection with other conditions than cancer, including some autoimmune disorders and infectious diseases.

This overview will focus on the structure, function and diagnostics of the human MDR-ABC transporters. The characterization of the function and drug-substrate interactions of the ABCG2 protein and its mutant variants will be presented. The talk will also include new data regarding the function and modulation of the human MRP1, preferentially transporting hydrophobic anionic conjugates, but also extruding hydrophobic uncharged drugs. In order to investigate the role of the highly conserved *signature* motifs in the ATP hydrolysis of MRP1, we prepared site-directed mutants designed on the basis of a bacterial ABC structure. The wild-type and mutant MRP1 proteins were expressed in Sf9 insect cells, and the catalytic activity was assayed by ATPase and vesicular transport experiments. ATP binding and occlusion were studied by using ^{32}P-8-azido-ATP, a labeled photoreactive ATP analog, and transition-state formation was investigated in the presence of phosphate-analog inhibitory anions. We found that the targeted amino acids have a key role in the regulation of the catalytic activity of MRP1. These studies may offer new targets for developing inhibitors of MRP1-dependent drug resistance.

CHALLENGES AND REWARDS FOR GENE THERAPY IN THE 21ST CENTURY

Kevin J. Scanlon

Keck Graduate Institute, Claremont, California 91711, U.S.A. E-mail: Kevin.Scanlon@kgi.edu

The field of Gene Therapy has made progress in the development of new promoters and novel delivery systems for the treatment of human disease. These new findings may ultimately be exploited for cancer gene therapy in collaboration with the future developments of stem cell

research. Conversely, challenges still remain before gene therapy will have a significant impact on cancer. The preclinical model systems for cancer lack the ability to predict the clinical reality of patient's treatment and response to therapy. This places the burden on the physicians to be more vigilant to observe subtle changes in patient response that were not observed in the preclinical models. Systemic gene therapy delivery requires further advances before the pharmaceutical industry has a marketable product. These delivery systems lack the ability to achieve pharmacological doses of therapeutic genes in the target tissue. Until these challenges are addressed, gene therapy will remain on the sidelines as a cancer modality. Yet, the field should be optimistic with the current progress. Recent advances in molecular genetics have introduced new tools for the manipulation of gene expression, including RNA-knockout technology. With an improved understanding of the molecular basis of disease, we have defined a plethora of new targets for anti-RNA-based therapy. The principal appeal of this interfering RNA therapy is its inherent simplicity. This is based on the hypothesis that antisense deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) hybridize to the target RNA in complementary Watson-Crick fashion, thereby resulting in sequence-specific suppressions of gene expression. However, a number of problems surfaced which threatened the theoretical appeal and presumed scientific mechanisms of action of these compounds. A clearer picture has emerged over time regarding the practical realities of anti-RNA targeting. The issues of non-sequence-specific and/or non-antisense activity need to be fully addressed before a particular antisense molecule is successfully applied to the clinical target. In conclusion, despite these challenges, new cancer targets will be characterized and their potential clinical efficacy demonstrated. These novel strategies will be discussed in the role of new therapeutics for cancer.

EXTRACELLULAR MATRIX PROTEINS PROTECT SMALL CELL LUNG CANCER FROM CHEMOTHERAPY-INDUCED APOPTOSIS: A MECHANISM FOR DRUG RESISTANCE *IN VIVO*

Tariq Sethi

Edinburgh University Medical School, Teviot Place, Edinburgh, EH8 9AG, Scotland. E-mail: tsethi@serv1.med.ed.ac.uk

SCLC, *in vivo*, is surrounded by an extensive stroma of ECM both at primary and secondary metastatic sites, which in part contains Fibronectin (Fn), Laminin (Ln) and collagen IV. The extent of ECM deposition around SCLC cells *in vivo* has not generally been appreciated. Evidence suggests that, *in vivo*, SCLC creates a specialised environment as a consequence of autocrine and paracrine effects which, using an inflammatory analogy, likens SCLC to "a wounding reaction" with the laying down and remodelling of ECM, growth factor release and neovascularisation.

Doxorubicin, cyclophosphamide, etoposide and cis-platinum cause a concentration-dependent stimulation of apoptosis in SCLC cells. The binding of SCLC cells to a number of ECM proteins found in increased amounts around SCLC *in vivo* reduces the cytotoxicity of these chemotherapeutic agents. This occurs as a result of β1 integrin-dependent adhesion to Ln, Fn and collagen IV. Despite anchorage-independence, binding of SCLC cells to ECM proteins confers protection from chemotherapy-induced apoptosis. Furthermore, Ln and collagen IV have been shown to be the predominant glycoproteins of basement membranes and induce migration probably by haptotaxis. This appears to play an important role in metastasis in a number of tumour cell lines. Thus, at primary and secondary metastatic sites, there appears to be a permissive environment for SCLC cell proliferation, protection from apoptosis and resistance to chemotherapy.

Our results suggest that the resistance to chemotherapy induced by β1-mediated adhesion to ECM is due to an increase in the level of tyrosine kinase activity. However, increased intracellular tyrosine phosphorylation had no effect on etoposide-induced topoisomerase II inhibition. Thus, despite chemotherapy-induced DNA damage, ECM-mediated tyrosine kinase activation blocks caspase activation which prevents apoptosis.

To determine the translational research implications of these findings, we undertook retrospective analysis of the available clinical. Sixteen patients who had received some standard form of chemotherapy were identified (8 had no or focal Fn matrix and 8 had extensive Fn matrix) aged 62.5 ± 2.3 yrs, having a survival time of 11.5 ± 1.7 months (mean ± SEM). There was no significant age or sex difference between the two groups. Patients with extensive matrix around their tumours had significantly shorter survival time from diagnosis 8.4 ± 1.7 months compared to patients with focal or no matrix 15 ± 2.6 months (mean ± SEM) $p<0.05$ (paired Student's *t*-test).

Our *in vitro* and *in vivo* data provide strong preliminary evidence that adhesion to ECM proteins play a critical role in SCLC cell resistance to chemotherapy. Cancer cells bound to ECM may escape chemotherapy-induced cell death and then with subsequent genetic damage drug-resistant clones are selected. We believe this is an excellent model to explain SCLC behaviour *in vivo* and why partial responses and local recurrence of SCLC are often seen after chemotherapy. Novel therapeutic strategies for SCLC must take into account this mitogenic complexity.

LIPOSOME-BASED THERAPY OF A TAXANE-RESISTANT BRAIN TUMOR MODEL

Robert M. Straubinger[1], Rong Zhou[2], Robert D. Arnold[1], Jeanine E. Slack[1] and Richard V. Mazurchuk[2]

[1]Depts. of Pharmaceutical Sciences, University at Buffalo, NY; [2]Molecular and Cellular Biophysics, Roswell Park Cancer Institute, NY; [3]Currently: Dept. of Radiology, University of Pennsylvania, Philadelphia, U.S.A. E-mail: rms@acsu.buffalo.edu

Aims: Microparticulate drug carriers such as liposomes can improve therapy of drug-resistant tumors through several effects, including increased tumor drug deposition, decreased toxicity, or pharmacokinetics that are more optimal for eliciting tumor killing. Our aim was to investigate the therapeutic potential of liposome-encapsulated paclitaxel and doxorubicin against an advanced intracerebral brain tumor model. We also investigated mechanisms of action that may be exploitable in designing liposome-based therapeutic regimens. *Methods*: Paclitaxel was incorporated at 3 mol% in 0.1 μM liposomes (PAC-L) of phosphatidylcholine: phosphatidylglycerol (9:1 mole:mole). Doxorubicin was encapsulated in long-circulating liposomes (DXR-L; distearoyl phosphatidylcholine: cholesterol:polyethyleneglycol- phosphatidylethanolamine, 9:5:1) using a "remote-loading" procedure. Brain tumors were established in Fisher 344 rats by stereotaxic implantation of $4x10^4$ rat 9L gliosarcoma cells into the caudate/putamen; therapy was initiated 8 days after tumor implantation. Lifespan and toxicity were monitored, along with drug deposition and tumor vascular permeability. *Results*: The 9L tumor expresses the multidrug resistance-associated p-glycoprotein and is moderately resistant to taxanes. PAC-L given at 20 mg/kg on days 8, 11 and 15 mediated the greatest increase in lifespan (ILS) we have observed in this model (40% ILS) and was well-tolerated. Higher dose intensities at less frequent intervals were less efficacious (1). No dose of free paclitaxel (similar to the Taxol® formulation) prolonged lifespan and dosing equivalent to the most efficacious liposomal regimen accelerated death due to toxicity. Previously we observed that DXR-L given in 3 weekly doses of 5.7 mg/kg mediated a 29% ILS (2). Free DXR at the same dose and schedule accelerated death. Breakdown of the tumor vascular permeability barrier was observed with DXR-L, but not with free DXR, PAC-L, or free paclitaxel (3). Drug deposition and vascular permeability increased markedly with repetitive DXR-L treatments. *Conclusion*: Liposome-drug complexes provide a functional approach to overcome drug resistance through a variety of effects. Lower, sustained levels of paclitaxel, as provided by repetitive doses of liposomal drug, appeared superior in this relatively drug-resistant brain tumor model. Free paclitaxel was not efficacious in any dose or regimen tested. Doxorubicin liposomes, but not free drug or PAC-L, exhibited a clear anti-vascular effect in tumor. These drug-carrier-mediated effects may be exploitable, alone or in combination, to improve therapy of challenging tumor targets.

References

1 Zhou R, Mazurchuk RV, Tamburlin J and Straubinger RM: Efficacy of liposomal paclitaxel in an intracranial rat brain tumor model. *Submitted* (2003s).

2 Sharma US, Sharma A, Chau RI and Straubinger RM: Liposome-mediated therapy of intracranial brain tumors in a rat model. Pharm Res *14*: 992-998, 1997.

3 Zhou R, Mazurchuk RV and Straubinger RM: Antivasculature effects of doxorubicin-containing liposomes in an intracranial rat brain tumor model. Cancer Res *62*: 2561-2566, 2002.

CLINICALLY RELEVANT CELLULAR MODELS OF RADIO- AND CHEMO- RESISTANCE

Ross A. Davey

Bill Walsh Cancer Research Laboratories, Royal North Shore Hospital. St Leonards 2065 Australia. E-mail: rdavey@med.usvd.edu.au

Resistant sublines of the H69 human small cell lung cancer (sclc) cells were developed using low doses of cisplatin, etoposide, epirubicin and fractionated radiation. These sublines show low, but stable resistance (1,2). Previously, we have shown that the resistance of the H82sclc cells was reversed by treatment with low dose taxol (3). *Aim*: Our aim was to determine the molecular changes associated with resistance in the H69 cells and the effect of low dose taxol on resistance and protein expression. *Methods*: Gene expression was determined using Northern blot, Western blot and 2-dimensional protein electrophoresis/mass spectrometry in the H69 cells, H69-CP (treated with 100ng/ml cisplatin), H69-EPR (treated with 40ng/ml epirubicin), H69-VP (treated with 500ng/ml etoposide) and H69/R38 (treated with 37.5Gy fractionated X-rays) sublines. Cells were treated with 10ng/ml taxol and changes in resistance and protein expression were determined. The response to 4 Gy X-ray treatment of the H69 and H69/R38 cells was also determined using subtractive hybridisation. *Results*: Resistance in the H69-CP subline (3-fold to cisplatin) was predominantly associated with changed glutathione metabolism, in the H69-VP subline (2-fold to etoposide) with decreased topoisomerase IIa and in the H69-EPR (7-fold to epirubicin) with increased MRP1. In

contrast, several changes in the H69/R38 were identified, including increased topoisomerase IIa and decreased glutathione-S-transferase. Although these changes in the H69/R38 cells were in the opposite direction to those in the drug-derived sublines, the H69/R38 cells were resistant to cisplatin (13-fold) and daunorubicin (6-fold). The change in gene expression associated with stable resistance in the H69/R38 subline was similar to that caused by irradiating the H69 cells with 4Gy X-rays. In addition, there were changes in oncogene expression. Bcl-2 expression was no longer detectable in the H69-CP and H69-EPR sublines and was dramatically decreased in the H69/R38 subline while the H69-VP subline showed increased bcl-2 expression. N-myc, which is often overexpressed in sclc, was no longer detectable in the H69-CP and H69-EPR cells but was increased in the H69/R38 cells. 2-dimensional protein electrophoresis/mass spectrometry also identified changes in metabolic enzymes, cytoskeletal, stress and redox-associated proteins in the resistant sublines. Treatment with taxol, which reversed resistance only in the drug-selected sublines, also reversed many of the protein changes associated with the development of resistance. *Conclusion*: These models suggest that repetitive treatment may promote resistance by stabilising the transient changes in expression associated with the initial cellular response. The sensitisation of drug resistance by taxol suggests a potential role for its use in treatment of resistant sclc.

References

1 Henness S, Davey MW, Harvie RM and Davey RA: Int J Rad Oncol Biol Phys *54*: 895-902, 2002.
2 Locke V, Davey RA and Davey MW: Anti-Cancer Drugs *in press*, 2003.
3 Su GM, Davey MW and Davey RA: Int J Cancer *76*: 702-708, 1998.

DRUG RESISTANCE IN RESPONSE TO TREATMENT WITH LOW DOSES OF DRUG

Mary W. Davey, Lauren A. McCann, William J. Booth, Karen V. Cullen and [1]Ross A. Davey

Dept. Cell & Molecular Biology, University of Technology, Sydney, Gore Hill NSW 2065; [1]Bill Walsh Cancer Research Laboratories, Royal North Shore Hospital, St Leonards NSW 2065, Australia.

Understanding drug resistance mechanisms is important to the continued success of cancer treatment. Identification of the molecular changes resulting from treatment with low, non-cytotoxic doses of drug is important as it is these surviving cells that potentially lead to clinical resistance. Our aim therefore is to understand the development of resistance to epirubicin and cisplatin in the CCRF-CEM (CEM) leukaemia cells to provide model systems for the early response events to drug treatment. *Methods*: CEM cells were treated intermittently for 3-4 days with increasing doses of epirubicin (IC_{50} 58 ± 10 ng/ml) from 16 to 1000 ng/ml drug to produce the E series (1). To produce cisplatin-resistant cells, CEM cells were similarly treated with cisplatin (IC_{50} 540 ± 30 ng/ml) with 100 to 5000 ng/ml drug to produce the CP series. Resistance was determined after 4 days incubation in 2-fold serial drug dilution by the MTT viability assay. Daunorubicin accumulation was determined by flow cytometry. Cisplatin was determined by atomic absorption spectroscopy. *Results*: Treatment of CEM cells with 16 to 50 ng/ml epirubicin produced 5-fold resistance ($p<0.05$). These cells had decreased daunorubicin accumulation, which was not reversed by treatment with BSO. Treatment with epirubicin >100 ng/ml produced sublines with high drug resistance, significantly greater decreased daunorubicin accumulation and increased expression of the multidrug transport protein MRP1. There were no changes in glutathione S-transferase, or in cellular glutathione, however cells were slightly sensitised to cisplatin. While there was no change in the expression of topoisomerase IIa in the low level drug-resistant cells, there was decreased formation of cleavable complexes (>45 decrease at 2 uM etoposide; $p<0.05$) giving resistance to etoposide and the anthracyclines. In contrast to this, treatment of the CEM cells with cisplatin produced a series of drug-resistant sublines with increasing resistance to 1,600 ng/ml. Treatment at higher doses (up to 5 μg/ml) did not increase resistance to cisplatin. Cisplatin resistance correlated with increased intracellular glutathione (up to 2-fold; $p<0.05$), and was associated with significantly decreased cisplatin accumulation (>35; $p< 0.01$). The cisplatin-resistant cells had a low, but significant increase in daunorubicin accumulation (15; $p<0.05$) but no change in cytotoxicity with continuous drug exposure. *Conclusion*: These results suggest that changes in drug accumulation are an early response to treatment with drug doses well below their IC_{50} dose and provide models to examine molecular mechanisms and potential changes in cellular membranes that may lead to changes in drug accumulation after treatment with low doses of drugs.

References

1 Davey RA, Longhurst TJ, Davey MW *et al*: Drug resistance mechanisms and MRP expression in response to epirubicin treatment in a leukaemia cell line. Leuk Res *19*: 275-82, 1995.

THE ANTI-NEOPLASTIC AGENT ADRIAMYCIN ALTERS THE SUSCEPTIBILITY OF THE PATHOGENIC YEAST *CANDIDA ALBICANS* TO AMPHOTERICIN B

Kevin Kavanagh

Medical Mycology Unit, Department of Biology National Institute for Cellular Biotechnology, National University of Ireland Maynooth, Co. Kildare, Ireland. E-mail: kevin.kavanagh@may.ie

Cancer patients are susceptible to a range of fungal infections as a result of immuno-suppression due to the nature of their underlying condition or as a consequence of therapies implemented to treat their illness. The yeast *Candida albicans* is responsible for the majority of fungal infections in cancer patients and can cause a range of superficial and systemic diseases. Fungal infections are usually treated with azole (*e.g.* fluconazole) or polyene (*e.g.* amphotericn B) anti-fungal agents and can prove fatal. In this study we investigated the interaction of an anti-neoplastic agent, adriamycin, with *C. albicans* and sought to determine whether it promoted tolerance to amphotericin B.

Cultivation of *C. albicans* in the presence of adriamycin and amphotericin B leads to an increase in tolerance to the latter agent by a factor of almost 90%. We established that after prolonged exposure to adriamycin, this tolerance is lost. Cultivation of *C. albicans* in medium supplemented with adriamycin leads to a reduction in cellular respiration by 56%. This has the effect of suppressing the synthesis of ergosterol - a constituent of the fungal cell membrane which is a target for the amphotericin B. In addition, adriamycin-treated cells show elevated levels of the drug efflux pump CDR 1.

We postulate that adriamycin induces tolerance to amphotericn B by interacting with the process of oxidative phosphorylation and inhibiting respiration. One consequence of this is a reduction in the generation of NADPH which is required for the synthesis of ergosterol. Reduced levels of this sterol in the fungal cell membrane gives fewer binding sites for amphotericin B hence a higher concentration is required to achieve the death of the cell. The increased expression of drug efflux pumps may also serve to reduce the amount of toxic products within the cytoplasm and hence ameliorate the effect of the anti-fungal drug.

This work demonstrates that adriamycin anti-neoplastic therapy has the potential to increase the tolerance of *C. albicans* for amphotericin B by disrupting respiration which has an adverse effect on the ergosterol content of the fungal cell membrane and leads to elevated expression of CDR 1. This may have deleterious consequences for cancer patients and could lead to the appearance of amphotericin B-tolerant *C. albicans* infections in patients receiving adriamycin anti-neoplastic therapy. This work highlights the need to monitor the occurrence and treatment of fungal infections in cancer patients receiving adriamycin chemotherapy to ensure that anti-fungal tolerance does not emerge and compromise recovery.

Acknowledgements
This work was supported by funding from the HEA under PRTLI 3.

APOPTOSIS IN COLONIC TUMOURIGENESIS

E.F. Gaffney

Department of Histopathology, St James's Hospital and Trinity College, Dublin, Ireland

Background: One "hallmark of cancer" is considered to be evasion of apoptosis. Nevertheless, there is extensive apoptosis in cancer and apoptotic indices are higher in clinically aggressive tumours than in indolent malignancies. Although multiple potential apoptosis-inducing stimuli have been identified, the precise mechanisms that induce apoptosis in individual cancer types are not known. *Methods*: Colonic adenomas may show extensive apoptosis and we therefore examined the morphological patterns of apoptosis in H and E sections of 184 colonic adenomas and 14 adenocarcinomas, correlating findings with standard clinicopathological variables, including size and grade of dysplasia. Two patterens were identified and assessed semiquantitatively - confluent basal apoptosis (CBL) and crypt lumen apoptosis (CLA). *Results*: CBL was identified in 124 adenomas and was significantly associated with small adenomas <5mm ($p<0.0001$), whereas CLA was identified in 104 adenomas and correlated significantly with large adenoma size ($p<0.001$) and high grade dysplasia ($p<0.0003$). Adenomas showing both patterns (51) were significantly larger ($p<0.0001$), and the CLA pattern was very prominent in adenocarcinomas. *Conclusion*: Two distinctive morphological patterns of apoptosis during early and late colonic tumourigenesis suggest different apoptosis induction mechanisms or different efffector pathways, *i.e.* an "apoptosis pattern switch". This is being investigated further using a proteomic approach.

Abstracts of Poster Presentations

EFFECT OF BrdU ON REPORTER GENE EXPRESSION IN TRANSIENTLY TRANSFECTED LUNG CANCER CELL LINE.

Aisling Pierce, Niall Barron and Martin Clynes

National Institute for Cellular Biotechnology, Dublin City University, Dublin 9, Ireland. E-mail: aisling.pierce4@mail.dcu.ie

The poorly-differentiated lung cancer cell line, DLKP was transiently transfected with plasmids containing MRP-1 promoter fragments (containing varying lengths of the MRP-1 promoter upstream of the transcriptional start site) linked to a luciferase reporter gene. The effect of BrdU treatment on MRP-1 promoter activity was investigated using a Promega Luciferase Reporter Assay™ system and by RT-PCR for luciferase mRNA, in order to identify potential drug-responsive sites within the MRP-1 promoter. An approximate 3-fold increase in luciferase enzyme activity was observed in the MRP-1-transfected BrdU-treated samples. However, BrdU treatment caused a similar increase in luciferase enzyme activity in the pGL control and pGL basic samples, which should act as controls for the luciferase enzyme assay. Treatment of cells transfected with the pMRP-1 –411 plasmid with 10μM BrdU saw a further 3-fold increase over the 1μM BrdU-treated sample. mRNA levels remained unchanged (as assessed by RT-PCR) for luciferase and reflected no increased gene expression in response to BrdU treatment. This would suggest that, in transiently transfected cells, BrdU treatment exerts its effect at a post-transcriptional level.

INHIBITION OF INVASION AND MIGRATION BY CONJUGATED LINOLEIC ACID IN BREAST CANCER CELL LINES

Aisling Redmond, David Murray, Rosaleen Devery, [1]Catherine Stanton and Susan McDonnell

School of Biotechnology, National Institute for Cellular Biotechnology, Dublin City University; [1]Teagasc Dairy Products Research Centre, Moorepark, Fermoy, Co. Cork, Ireland. E-mail: aisling.redmond8@mail.dcu.ie

Aims: The majority of cancer patients die because their tumour has metastasised to distant sites in the body. The identification of therapies aimed at treating metastatic disease is important. Many *in vitro* and *in vivo* studies have shown that conjugated linoleic acid (CLA), a polyunsaturated fatty acid found in natural food sources has anti-cancer activity. The aim of this study was to investigate the effect of a mixture of CLA and its purified isomers on the invasion and migration of a murine breast carcinoma cell line, 4T1. We also looked at the effect of these lipids on the expression of matrix metalloproteinases (MMPs) and their inhibitors the tissue inhibitors of metalloproteinases (TIMPs). *Methods*: 4T1 cells were cultured under standard conditions and cytotoxicity was determined using the MTS cell proliferation assay for a CLA mix, containing various isomers, for the purified predominant isomers present in CLA, *c*9,*t*11 and *t*10,*c*12, and for linoleic acid (LA). Invasion assays were performed using BD Biocoat matrigel invasion chambers and migration assays using Falcon cell culture inserts. Gelatin zymography was used to examine MMP expression and reverse zymography for TIMP expression. Cells were treated with 10μg/ml CLA/purified isomers/LA for 24 hours and the media was then collected and concentrated using Amicon microcon filters. *Results*: Initial experiments were performed to determine the IC_{50} values (inhibitory concentration that killed 50% of the cells) of the CLA mix, individual purified isomers and LA using a range of concentrations over 24 h, 4 days and 7 days. For migration and invasion assays, 4T1 cells were treated with a sub-lethal dose of CLA, 10μg/ml, for 24 h. Both the CLA mix and the individual purified isomers were shown to decrease the invasion and migration of the 4T1 cells with the *t*10*c*12 having the most potent effect, decreasing invasion by 87% and migration by 39%. Interestingly, it appeared that whilst the LA caused an increase in the migration of the cells it had no effect on the invasion. The expression of MMPs and TIMPs were examined using zymography and reverse zymography. The 4T1 cells constitutively expressed MMP-9 which was not modulated by treatment with CLA and its isomers. In contrast the cells also expressed TIMP-1, which was down-regulated by treatment with CLA, its purified isomers and LA. *Conclusion*: These results indicate that CLA may have potential as an anti-metastatic agent. It is hoped that this research will contribute towards elucidating the mechanisms involved in the anti-invasive effect of CLA and its purified isomers. Future work will examine the *in vivo* effects of this fatty acid in a murine model of metastatic breast cancer.

IN SILICO DATA MINING CAN IDENTIFY NOVEL GENES OVEREXPRESSED IN COLONIC NEOPLASIA

Alan Moss[1], Stephen Madden[1], Anne-Marie Mulligan[2], Conor O Keane[2], Hugh Brady[1], Padraic Mac Mathuna[3] and Peter Doran[1]

Human Genomics & Bioinformatics Research Unit, [1]Department of Medicine, University College Dublin; [2]Department of Pathology & [3]Gastrointestinal Unit, Mater Misericordiae University Hospital, Dublin, Ireland. E-mail: moss.genome@ mater.ie

Background: Many of the genetic pathways involved in the development of colonic neoplasia in humans are unknown. Digital Differential Display (DDD) is a computational strategy for the identification of cDNAs from public sequence databases whose expression is altered in different pathological states (1). Here we describe the application of a high-throughput annotation pipeline to analysis of colonic neoplasia tissue libraries. *Aims*: In this study we have employed an integrated bioinformatics-based approach to: (a) identify genes whose expression is altered in colon cancer libraries; (b) annotate cDNAs without homology to known genes that are identified as disease-associated genes; (c) validate the technique in *ex vivo* specimens. *Methods*: cDNA libraries from normal and neoplastic colon were compared using Digital Differential Display. Transcripts without homology to known genes were annotated using a novel bioinformatics platform, Digital Extractor, which was developed in-house. RT-PCR was used to detect a sample number of the identified sequences in RNA extracted from *ex vivo* specimens. *Results*: DDD comparison of colon cancer libraries to normal colon and normal adult tissue identified 204 ESTs altered in colon cancer; 38 of these genes have previously been described in colon cancer (*e.g.* GPA33), 127 represent known genes that have not previously been identified in colon cancer (*e.g.* MYEOV). Furthermore, 39 cDNAs without homology to known genes were identified. Annotation of these data resulted in the identification of several known genes (*e.g.* CDX2) and hypothetical proteins. RT-PCR in *ex vivo* specimens confirmed the up-regulation of GPA33, and MYEOV, a gene not previously described in colon cancer. *Conclusion*: This novel computational biology-based approach can identify genes differentially expressed in colon cancer. The technique has been validated *ex vivo*. This may provide a novel tool to identify markers of neoplasia.

References

1 Scheurle D, DeYoung MP, Binninger DM, Page H, Jahanzeb M and Narayanan R: Cancer Res *60*: 4037-4043, 2000.

ANTI-ANGIOGENIC GENE THERAPY FOR CANCER: HYPOXIA-INDUCIBLE EXPRESSION OF MATRIX METALLOELASTASE *IN VITRO* AND *IN VIVO*

Angela Duffy, Brian Gibson, David Bouchier-Hayes and Judith Harmey

Dept. of Surgery, ERC/R.C.S.I. Building, Beaumont Hospital, Dublin. 9, Ireland. E-mail: aduffy@rcsi.ie

With the major advances in genetics, many researchers are exploring gene therapy to treat many diseases, including cancer. However, the lack of specificity associated with many gene therapy approaches remains a major drawback. This project aims to develop tumour-specific anti-angiogenic gene therapy. Multiple areas of hypoxia exist in tumours (1) and we plan to exploit this fact to achieve tumour-specific expression of a therapeutic gene. Tumour vascularisation, growth and metastasis are dependent upon angiogenesis. Angiogenesis is regulated by a balance of pro- and anti-angiogenic factors (2). In tumours this balance is tipped towards angiogenesis, facilitating growth and formation of new blood vessels and tumour progression. We aim to tip the balance towards anti-angiogenesis by overexpressing the anti-angiogenic gene matrix metalloelastase, (MME), within tumours. Active MME cleaves plasminogen to produce angiostatin, a naturally occurring endogenous inhibitor of angiogenesis (3). Angiostatin is secreted from cells, thus, a 'bystander' effect should be achieved. We have constructed a plasmid vector with the MME gene under a hypoxia-inducible promoter using the commercial pGL3 (Promega) promoter vector (lacking enhancer elements to drive expression). A trimer of hypoxia response elements were cloned upstream of the minimal SV40 promoter. The luciferase reporter gene that is driven by these promoter/enhancer sequences was replaced with MME. The MME gene was tagged with a c-myc epitope to distinguish it from endogenous MME. Insertion of a constitutive expression cassette, encoding Neomycin resistance, downstream of the MME polyadenylation sequence permits selection of transfected cells. A corresponding control vector was also generated, lacking the MME gene, but containing HRE and Neo cassette. Mammary adenocarcinoma 4T1 cells were transfected with either the MME plasmid or the control plasmid. Transfected cells were then exposed to normoxia or hypoxia (0.5-1% O_2) for various timepoints between 6 and 24 hours. Expression of the soluble MME/c-myc fusion protein in the cell culture supernatants was determined by immunoprecipitation with an anti-MME antibody and Western blot analysis using an anti-c-myc antibody. Expression of the MME/c-myc fusion protein was observed in 4T1 cells transfected with the MME plasmid exposed to hypoxia for 18 and 24 hours. MME/c-myc expression was not observed in control-transfected 4T1 cells or under normoxic conditions.Our ultimate aim is to examine the effect of MME overexpression on tumour progression '*in vivo*'. Mammary adenocarcinoma 4T1 cells transfected with the MME or control plasmid will be injected into the mammary fad pad of Balb/c mice. The effect of MME overexpression on tumour growth will be established.

References

1 Vaupel P, Kallinowski F and Okunieff P: Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumours: a review. Cancer Res *49*: 6449-6465, 1989.

2 Hanahan D and Folkman J: Patterns and emerging mechanisms of the angiogenic switch during tumourigenesis. Cell *86*: 353-364, 1996.

3 O'Reilly MS, Holmgren L, Chen C and Folkman J: Angiostatin induces and sustains dormancy of human primary tumours in mice. Nature Medicine *1*: 149-153, 1995.

CHEMOSENSITISATION OF TUMOUR CELL-LINES WITH PEPTIDE INHIBITORS OF VEGF

Annemarie Byrne, D.J.Bouchier Hayes and J. Harmey

Education and Research Centre, Beaumont Hospital, Dublin 9, Ireland. E-mail: annembyrne@rcsi.ie

Background: Although Vascular Endothelial Growth Factor (VEGF) was originally identified as an endothelial cell-specific mitogen, there is increasing evidence that VEGF has an autocrine effect on tumour cells. Several VEGF receptors have been identified, Flt-1, Flk-1/KDR and Neuropilin-1. *Aim*: To establish the anti-tumor efficacy of peptide inhibitors of VEGF alone and in combination with conventional chemotherapy. *Methods*: We treated 4T1 murine mammary adenocarcinoma cells and RENCA Kidney carcinoma cells with the chemotherapy drugs, 5 Fluorouracil (5FU), Cisplatin and Taxotere. Proliferation was determined by the MTT proliferation assay. 4T1 and RENCA cell lines were treated with peptides directed against two of the receptors of VEGF, Neuropilin-1 and Flk-1/KDR. The effect of these peptides in combination with chemotherapy on cell growth and apoptosis were assayed by the MTT proliferation assay and by a Caspase-3 apoptosis assay. *Results*: 4T1 cells were sensitive to Taxotere at 5 and 10nM, Cisplatin at 2.5 and 5 μg/ml and 5 FU at 1μM and 10μM. RENCA cells were sensitive to Taxotere at 10nM and Cisplatin at 2.5 μg/ml (significant ($p<0.05$) and resistant to 5FU even at 50 μM. The anti-NRP-1 peptide decreased proliferation of 4T1 cells but did not have effect apoptosis of RENCA cells. The anti-Flk-1 peptide had no effect on the apoptosis of either cell line. Combining 5nM taxotere or 2.5 μg/ml Cisplatin with the anti-NRP-1 peptide had a significant effect on the 4T1 cell proliferation compared to chemotherapy or peptide alone. (Anti-NRP-1 alone: ~12 % 5nM Taxotere: ~15% decrease, 5nM Taxotere +anti-NRP-1: ~25% decrease, 2.5 μg/ml Cisplatin: ~20% decrease, 2.5 μg/ml Cisplatin + anti-NRP-1 : ~40% decrease) (all significant ($p<0.05$)). *Conclusion*: 4T1 cells express the Flt-1 and NRP-1 receptor. Blocking the NRP-1 with peptides and chemotherapy combined had a greater effect on proliferation than using either alone. Blocking NRP-1 may be of benefit in increasing tumour cell sensitivity to chemotherapy drugs.

MDR-3 PGP EXPRESSION IN INVASIVE BREAST CANCER

Annemarie Larkin[1], Elizabeth Moran[1], Susan Kennedy[2], Aine Adams[1] and Martin Clynes[1]

[1]National Institute for Cellular Biotechnology, Dublin City University, Glasnevin Dublin 9; [2]Department of Pathology, Royal Victoria Eye & Ear Hospital, Adelaide Rd., Dublin 2, Ireland. E-mail: Anne-Marie.Larkin@dcu.ie

MDR-3 Pgp has been shown to transport several cytotoxic drugs including digoxin, pacitaxel, daunorubicin, vinblastine and ivermectin. This Pgp has been found to be associated with drug resistance in certain B-cell leukaemias and to be responsible for drug resistance in an MDR-3 transformed yeast strain. However the role of MDR-3, if any, in breast cancer and other solid tumours remains unknown. An immuno-histochemical study of a panel of invasive breast carcinomas was carried out using an MDR-3 specific monoclonal antibody (MAb), 6/1G, in order to address any possible prognostic or predictive role for MDR-3 Pgp in human breast cancer. Preliminary results indicate that MDR-3 Pgp was expressed, to varying extents, in the majority (73%) of invasive tumours studied. To our knowledge this is the first report of MDR-3 Pgp expression in breast tumour tissue. A small number of post chemotherapy treated tumours were studied. MDR-3 levels did not appear to increase following chemotherapeutic treatment suggesting that MDR-3 expression was not associated with exposure to chemotherapeutic drugs. Furthermore, MDR-3 expression did not appear to correlate with known patient or tumour characteristics. The exact significance of this expression and the contribution, if any, of MDR-3 Pgp in the drug resistance of breast cancer or other solid cancers warrants further investigation.

PAPP-A REGULATES IGF1 BIOACTIVITY *VIA* CLEAVAGE OF IGFBP4

Anthony Ryan, Dermot McBrierty, David Bouchier-Hayes and Judith Harmey

Royal College of Surgeons in Ireland, Dept. Surgery, Education and Research Centre, Beaumont Hospital, Dublin 9, Ireland. E-mail: Aryan@rcsi.ie

Insulin like growth factors (IGFs) play key roles in growth and development, cellular transformation, survival and growth of malignant cells. Bioavailability of IGFs is regulated by the IGF binding proteins (IGFBPs) which bind IGFs. IGF release is dependent on cleavage of the IGFBPs by the metalloprotease pregnancy-associated plasma protein A (PAPP-A). There is conflicting data on the significance of IGF and IGFBP expression in tumour growth. We examined the expression of IGFBP4 and its recently identified protease, PAPP-A, in normal and tumour mouse tissues and 4T1.2 mammary adenocarcinoma cells by Western blotting. We examined the effects of wild-type IGF1 and recombinant mutant IGF1, which is resistant to IGFBP binding, on proliferation of 4T1.2 cells.

4T1.2 cells secreted IGFBP4 but not the protease, PAPP-A. IGF1 given in doses of 50, 100, 500, 1000 and 2000ng/ml did not stimulate proliferation of 4T1.2 cells, whereas rmIGF1 50, 100, 500, 1000 and 2000ng/ml, which does not bind with IGFBP4, increased proliferation of these cells ($p>0.05$ ANOVA with LSD *post hoc*). In normal tissues, intact IGFBP4 expression was highest in brain tissue, which did not express PAPP-A. In normal spleen, liver, lung, heart, skin and kidney, high levels of PAPP-A were expressed and IGFBP4 was either present at very low levels or completely absent. In 4T1.2 mammary fat pad tumours, lung metastases and bone metastases, the pattern of intact IGFBP4 was the inverse of PAPP-A expression. These data show that IGFBP4-resistant IGF1 stimulates tumour cell proliferation, whereas IGF1 bound by IGFBP4 does not. Furthermore, intact IGFBP4 is only present in tissues where PAPP-A is absent. Our results suggest that PAPP-A regulates IGF1 bioactivity *via* cleavage of IGFBP4. Therefore, development of a protease-resistant IGFBP4, which could bind but not release IGF1 or by inhibiting production of PAPP-A, may be of therapeutic value in blocking the activity of IGF1 in breast cancer.

Acknowledgements
Funded by the Irish Cancer Society / Cancer Research Ireland.

AUGMENTATION OF ANTITUMOUR EFFICACY OF CHEMOTHERAPY IN A MURINE MODEL OF LEWIS LUNG CARCINOMA USING ERYTHROPOIETIN

Aoife M. Shannon, Claire M. Condron, David J. Bouchier-Hayes and Deirdre M. Toomey

Department of Surgery, Royal College of Surgeons in Ireland Education & Research Centre, Beaumont Hospital, Dublin 9, Ireland. E-mail: Ashannon2@rcsi.ie

Erythropoietin (Epo) is a glycoprotein hormone stimulator of erythropoiesis currently administered to cancer patients to treat chemotherapy-associated or tumour-associated anaemia. Epo is now thought to have other functions, such as a role in angiogenesis (1) and in the promotion of an antitumour immune response (2). Hypoxia occurs in areas of tumours with an inefficient vascular supply, leading to chemotherapeutic resistance (3). Many drugs have greater efficacy under oxygenated conditions. Anaemia is a contributory factor to hypoxia. The hypothesis of this project is that the administration of Epo to tumour-bearing mice in combination with chemotherapy will correct anaemia and reduce tumour hypoxia, ultimately resulting in improved therapeutic outcome. *Methods*: C57/Bl6 mice bearing a Lewis lung carcinoma (LLC) flank tumour were randomised into a control group, a group receiving chemotherapy (3 mg/kg cisplatin and 60 mg/kg gemcitabine) for 12 days, or chemotherapy combined with a once weekly dose of Epo (10 μg/kg darbepoetin alfa) beginning ten days before chemotherapy. Mice were anaesthetised using halothane. The chemotherapy drugs were administered according to a metronomic schedule designed to achieve maximal efficacy of the drug combination. End-points measured were tumour volume, blood haemoglobin concentration (Hemocue haemoglobin analyser) and proliferating tumour cells (proliferating cell nuclear antigen (PCNA) immunohistochemistry). In an *in vitro* study the effects of cisplatin and gemcitabine on LLC cell proliferation under normoxic and hypoxic (1% oxygen) conditions were measured using MTT assays. *Results*: The MTT assays showed that the chemotherapy drugs have greater efficacy under normoxic conditions. In the *in vivo* study, PCNA immunohistochemistry on the tumour sections showed that tumour cell proliferation was significantly reduced in the chemotherapy-treated groups when compared to the untreated control group. Chemotherapy-induced anaemia (haemoglobin <12 g/dl) was corrected by weekly Epo treatment, beginning ten days before chemotherapy. In this Epo-treated group, tumour growth was attenuated more effectively than in the non Epo-treated group. *Conclusion*: The delay in tumour growth in the Epo-treated group may be due to increased oxygenation within the tumour environment, resulting in improved efficacy of the chemotherapeutic drugs.

References
1 Anagnostou A *et al.*: Erythropoietin has a mitogenic and positive chemotactic effect on endothelial cells. PNAS USA *87*: 5978-5982, 1990.
2 Mittelman M *et al.*: Erythropoietin induces tumour regression and antitumour immune responses in murine myeloma models. PNAS USA *98*: 5181-5186, 2001.
3 Shannon AM *et al.*: Tumour hypoxia, chemotherapeutic resistance and hypoxia-related therapies. Cancer Treatment Reviews, in press.

ACTIVITY OF NOVEL PLANT EXTRACTS AGAINST MEDULLARY THYROID CARCINOMA-CELLS

Beate Rinner[1], Veronika Siegl[1], Peter Pürstner[2], Bernd Gesslbauer[3], Andreas Kungl[3], Thomas Efferth[4], Brigitte Brem[5], Harald Greger[5] and Roswitha Pfragner[1]

Departments of [1]Pathophysiology, [2]Obstetrics and Gynecology, [3]Pharmaceutical Chemistry and Pharmaceutical Technology, University of Graz, Austria; [4]Center of Molecular Biology, University of Heidelberg,, Germany; [5]Department of Comparative and Ecological Phytochemistry, Institute of Botany, University of Vienna, Austria. E-mail: roswitha.pfragner@uni-graz.at.

Medullary thyroid carcinoma (MTC) is a rare calcitonin-producing tumor, derived from the parafollicular C-cells of the thyroid. MTC occurs sporadically, familial (FMTC), or as part of the inherited cancer syndromes Multiple Endocrine Neoplasia type 2A (MEN2A) and type 2B (MEN2B). MTC is inherited in 25-30% of all cases due to mutation in the *ret*-proto-oncogene, located on chromosome 10 (10q11.2). Presently the only available therapy of MTC is surgical treatment, as MTC is known to be relatively insensitive to chemo- or radiation- therapy.

Five continuous cell lines (MTC-SK, SINJ, GRS-IV, GRS-V, BOJO) were established earlier from sporadic MTCs. An up-regulation of bcl-2 was found in each cell line. We investigated the activities of the genus *Stemona* (Stemonaceae), *Aglaia* (Meliaceae) and *Artemisia* (Asteraceae) on proliferation and apoptotic rates. Extracts have been used in traditional Chinese medicine, however no experience on their effects on medullary thyroid carcinomas has been reported so far. The genus *Aglaia* is mainly characterized by the presence of different types of flavaglines, whereas *Stemona* species accumulate typical alkaloids (1) with a pyrrolo[1,2-a]azepine nucleus and stilbenoids (2). Artesunate (3) is a semi-synthetic derivative of artemisinin, an active sesquiterpenelacton of *Artemisia annua*.

The five MTC-cell lines were treated with different concentrations of each sample for 24, 48 and 72 hours. Growth kinetics and viability were examined using the Casy-1-Cell Counter & Analyser and the WST-based cytotoxicity assay. Apoptotic cells were detected by immunofluorescence, by DAPI staining and by flow cytometry.

After incubation with each sample, a strong antiproliferative effect was recognized in each *Aglaia* species and Artesunate, and an enhancement of apoptosis was provoked by *Stemona tuberosa*. In contrast to normal cells, the nuclei of apoptotic cells had highly condensed chromatin that was uniformly stained by DAPI. This could take the form of crescents around the periphery of the nucleus, or the entire nucleus could appear to be one or a group of featureless, bright spherical beads.

The activity of the novel plant extracts possibly offers a new approach towards successful chemotherapy of the so far chemo-resistant medullary thyroid carcinoma.

References

1 Brem B, Seger C, Pacher T, Hofer O, Vajrodaya S and Greger H: J Agric Food Chem *50*: 6383-6388, 2002.
2 Pacher T, Seger C, Engelmeier D, Vajrodaya S, Hofer O and Greger H: J Nat Prod *65*: 820-827, 2002.
3 Efferth T, Olbrich A and Bauer R: Biochem Pharmacol *64*: 617-623, 2002.

Acknowledgements

This investigation was supported by the Styrian Cancer Aid Society and the Franz Lanyar Foundation, Graz, Austria.

INVESTIGATION INTO THE ROLE OF TRANSLATIONAL REGULATION OF GENE EXPRESSION IN THE GROWTH AND DIFFERENTIATION OF A POORLY-DIFFERENTIATED HUMAN LUNG CARCINOMA CELL LINE

Brendan Power, Paula Meleady and Martin Clynes

National Institute for Cellular Biotechnology, Dublin City University, Dublin 9, Ireland. E-mail: Brendan.Power@dcu.ie

Little is known about the biological mechanisms controlling development and differentiation in normal and disease states of the lung. Much of the work to date has concentrated on transcriptional regulation of development and differentiation while few studies exist on the potential role of translational regulation of gene expression in these processes. The expression of a number of translation initiation factors is known to be altered in a wide range of cancers, and these factors are involved in the development and differentiation of various cell lineages. Recent studies have shown increased expression of various translation initiation factors in different types of lung cancer, suggesting a role for translation in the regulation of growth in these cells.

The DLKP cell line is an extremely poorly-differentiated carcinoma cell line that does not express keratin proteins or other epithelial markers, despite being of epithelial origin. Previous studies have shown that treatment of DLKP cells with the differentiation-modulating agent 5'-bromo-2'-deoxyuridine (BrdU) can induce the expression of keratins 8, 18 and 19 and increases β1-integrin at a post-transcriptional level. This post-transcriptional up-regulation by BrdU was found to coincide with increased expression and phosphorylation of the translation initiation factor,

eIF4E. BrdU treatment was also found to alter the adhesion of DLKP cells to components of the extracellular matrix.

Stable and transient transfections using wild-type and two mutated human eIF4E constructs (mutated at Ser^{209} and Ser^{209}/Thr^{210}) were performed to investigate the potential role of eIF4E in the regulation of growth and differentiation in DLKP cancer cells. Our results show that increased eIF4E protein expression in these cells induced expression of keratins 8 and 18 and of β1 and α2 Integrins and adhesion to extracellular matrix components was increased. Increased levels of c-Myc protein were also observed in eIF4E transfected cells. These results suggest that translational regulation plays an important role in this model of lung cell growth and differentiation.

A NOVEL GENE DELIVERY SYSTEM FOR MAMMALIAN CELLS

Brian Gibson

Dept Surgery, RCSI ERC, Beaumont Hospital, Dublin 9, Ireland. E-mail: bgibson@rcsi.ie

Although gene therapy holds great promise for the treatment of both acquired and genetic diseases, its development has been limited by practical considerations. One difficulty is the lack of specificity associated with many gene therapy approaches. Also, although many novel routes of gene delivery are under investigation, efficacy of delivery remains quite poor particularly with primary cells, with viral vectors still being the most efficient means of delivery. The safety of viral vectors and their immunogenicity greatly limit their use. The aim of this project was to explore the efficacy of a novel, safe, lipid-based delivery system, cochleates (Biodelivery Sciences International). These highly stable lipid bilayer structures are non-toxic and non-inflammatory. The molecules housed within the lipid bilayers, and the cochleates themselves, are protected from degradation by this unique tightly-packed, multilayer structure. Encochleated amphotericin B remained biologically active when delivered by various routes, including oral administration. Rhodamine-labelled cochleates were incubated with a murine mammary adenocarcinoma cell line (4T1), a macrophage-hybrid cell line (H36.12) and primary monocytes/macrophages. For all *in vitro* work cochleates and nanocochleates (the same composition as cochleates but smaller) were used at concentrations of 5 and 10 μg/ml. In general, the cochleates entered the 4T1 line more readily then the H36.12 line. At 6 hours, 39% of the 4T1 cells were positive for rhodamine and 23.1% of the H36-12 cells were rhodamine- positive at this time. Nanocochleates were very effective at entering the H36-12 cells. After just 1 hour incubation with the rhodamine nanocochleates 97% of H36-12 cells were rhodamine positive. 7.24% +/- 3.77 of human monocytes matured *in vitro* for 3 days followed by 24 hours activation with hIFN-γ were rhodamine-positive after 1 hour. After five hours, 33.3% of these monocyte-derived macrophages were positive for rhodamine.

51.45% +/- 15.62 peritoneal murine macrophages incubated for 1 hour *in vitro* were rhodamine-positive. Using nanocochleates, for 1-or 3-hour incubation resulted in 44.25% +/- rhodamine-positive primary peritoneal macrophages. Following thioglycollate elicitation to the peritoneal cavity, 100 μg of cochleates were injected to this area. Following lavage, 1hour later *in vivo* transfection rates were calculated at 8.41%+/-5.17. Intra peritoneal injection of 2 mg nanocochleates per mouse, resulted in *in vivo* transfection rates of 88.1%+/- 12.89. Both cochleates and nanocochleates are effectively taken up by both cell lines and primary murine and human monocyte/macrophages, both *in vitro* and *in vivo*, suggesting that cochleates may be suitable for transgene delivery to monocytes/macrophages. Future studies will use cochleates deliver a gfp (green fluorescent protein) reporter plasmid to evaluate efficiency of transgene delivery and duration of expression. This will be carried out using both *in vitro* and *in vivo* models.

INCREASED INVASION AND EXPRESSION OF MATRIX METALLOPROTEINASE-9 IN COLORECTAL CELL LINES BY A CD44-DEPENDENT MECHANISM

David Murray and Susan McDonnell

School of Biotechnology, National Institute for Cellular Biotechnology, Dublin City University, Dublin 9, Ireland. E-mail: David.Murray5@mail.dcu.ie

Aims: The aim of this study was to investigate interactions between the matrix metalloproteinases (MMPs) and CD44 in controlling tumour cell invasion and metastasis. The MMPs are a large family of proteolytic enzymes collectively capable of degrading the entire extracellular matrix (ECM) giving them well-established roles in tumour cell migration and invasion. CD44, the major cell surface receptor for hyaluronic acid (HA), is a multifunctional transmembrane adhesion molecule which also plays a key role in cell signalling. The multi-stage process of tumour cell metastasis involves initial attachment of cells to the ECM, degradation of the ECM and subsequent migration and detachment of cells from the ECM. Cell adhesion molecules (CAMs) and MMPs are therefore crucial to this process. In order to investigate interactions between MMPs and CAMs we have established a panel of human colon cell lines. The cell lines studied included a

primary human colon adenocarcinoma cell line SW480 and its lymph node metastatic derivative, SW620. The SW480 cells have been stably transfected with the gene for MMP-7 (SW480M7) and MMP-9 (SW480M9). *Methods*: Cell lines were cultured and maintained under standard conditions. The *in vitro* invasion and migration assays were carried out using Biocoat matrigel invasion chambers following the manufacturer's instructions. Cell adhesion assays were carried out on various ECM components and MTS. MMP expression was determined using substrate gel zymography, Western blot analysis and reverse transcription polymerase chain reaction (RT-PCR). *Results*: The *in vitro* invasive activities of the cell lines through Matrigel© were compared. The SW620 cells were twice as invasive as their parental SW480 and the MMP transfected cell lines SW480M9 and SW480M7 were, respectively, three times and seven times more invasive than the SW480. The *in vitro* migratory capacities of these cell lines were also compared. Twice as many SW480M7 migrated through eight-micron transwell inserts compared to SW480. Interestingly less SW620 cell migration was observed compared to the parental SW480. MMP-9 expression increased when the SW480M9 cells were grown on HA and collagen. In addition, SW480M9 cells cultured in the presence of a CD44 activating antibody resulted in a time-dependent increase in MMP-9 activity. Treatment of the cells with HA also resulted in increased invasion with the SW480M9 cells showing a 2-fold increase. The adhesive nature of these cells to plastic and various ECM components were also compared. The SW480M9 were shown to be the most adherent to plastic, HA and fibronectin and the SW480M7 slightly less adherent. The increased HA adhesion in the SW480M9 also correlated with increased expression of the 80kDa and 120kDa CD44. *Conclusion*: These results demonstrate that CD44 and MMP interactions are important in controlling tumour cell invasion and metastasis. This work will help elucidate the exact mechanisms of such interactions as well as highlighting their importance in the metastatic process.

INVESTIGATION OF THE EFFECT OF THE NSAID SULINDAC ON TAXANE INFLUX/EFFLUX IN THE A549 CELL LINE

Denis Collins, Robert O'Connor and Martin Clynes

National Institute for Cellular Biotechnology, Dublin City University, Dublin 9, Ireland. E-mail: denis.collins5@mail.dcu.ie

Multi-drug resistance (MDR) is a major obstacle in the successful treatment of cancer; MDR imparts the ability to negate the effects of a broad range of chemotherapeutic regimens to a cancer. Drug efflux pumps from the ATP Binding Cassettes (ABC) superfamily have been found to be significant contributors to MDR. These include P-gp and the MRP family. Previous studies have shown that certain NSAIDs, including Sulindac, have the ability to potentiate the cytotoxicity of certain chemotherapeutic agents, many of which are known MRP1 substrates, in MRP1-expressing cell lines. Sulindac combined with epirubicin is presently part of an ongoing clinical trial.

The taxanes, Taxol (paclitaxel) and Taxotere (docetaxel), are reported P-gp substrates but it is unknown if their combination with an NSAID results in increased cell toxicity in the lung carcinoma cell line, A549. A549 expresses MRP1 and MRP4 at the mRNA level. We used combination toxicity assays and influx/efflux assays to look at two factors and their relationship, *i.e.*:

1. any possible potentiation of toxicity of the taxanes by Sulindac and
2. the physical dynamics of taxane transport in the A549 cell line.

We have also investigated the expression of MRP1 and MRP4 protein. We report the changes observed in taxane influx and efflux due to the presence of Sulindac.

MDR 1 AND MRP1 GENE EXPRESSION DURING DIFFERENTIATION OF MOUSE ES CELLS

Eadaoin Mc Kiernan, Lorraine O'Driscoll and Martin Clynes

National Institute of Cellular Biotechnology, Dublin City University, Dublin 9, Ireland. E-mail: eadaoin0@yahoo.com

Background: The MDR1 gene product, P-glycoprotein and multidrug resistance protein (MRP1) are associated with multidrug resistance in several cancer cell lines. Expression of these molecules has also been found in human normal tissue. Recent studies have focused on gaining more insight into the physiological role of ABC transporters. Analysis of MDR1 and MRP1 expression in porcine oocyte indicate that both genes are expressed in the oocyte at a transcriptional level. *Aim*: The aim of this study was to determine if mouse embryonic stem cells express MDR1 and MRP1 and subsequently to ascertain the effect of differentiation of these cells on MDR1 and MRP1 expression. *Method*: Murine ES-D3 cells were cultured on gelatin in the presence of leukaemia inhibitory growth factor (LIF). The cells were then induced to form embryoid bodies (EBs). A 3-step differentiation protocol was followed which directed the cells along an endodermal lineage. Total RNA was extracted from the cells at various stages throughout the differentiation procedure. Murine MDR1 and MRP1 specific primers were designed and used for RT-PCR analysis. Western blotting technique was used to

analyse protein extracted from both undifferentiated ES-D3 cells and embryoid bodies. *Results*: RT-PCR analysis showed MDR1 and MRP1 expression in undifferentiated ES-D3 cells. Expression of both molecules, at a transcriptional level, was down-regulated upon differentiation. Preliminary Western blotting results indicate that MDR1 is expressed in undifferentiated ES-D3 cells and is down-regulated upon differentiation to form embryoid bodies. MRP1 expression was not detected by Western blotting. *Conclusion*: MDR1 and MRP1 may have a physiological role to play in maintaining the pluripotency of embryonic stem cells and in maintaining the cells in their undifferentiated state.

Acknowledgements
This work was supported by funding from the Higher Educational Authority's PRTLI programme, Ireland.

DETECTION OF AMPLIFIABLE mRNA FROM CONDITIONED MEDIA OF CULTURED HUMAN CANCER CELLS

Elaine Kenny, Lorraine O'Driscoll and Martin Clynes

National Institute for Cellular Biotechnology, Dublin City University, Dublin 9, Ireland. E-mail: Elaine.kenny3@mail.dcu.ie

Background: A limited number of recent studies have indicated that it is possible to amplify extracellular mRNA from the serum of cancer patients (1,2). However, the number of serum specimens and the range of mRNAs analysed, to date, have been quite limited. It is important, therefore, to establish, in a well-controlled environment, if cancer cells routinely secrete gene transcripts detectable by RT-PCR. *Aim*: The aim of this study was to (i) establish if it is possible to routinely extract and amplify extracellular mRNA from the conditioned media of cultured human cancer cells, and (ii) investigate if any known tumour-related mRNAs are expressed and, if so, at what levels. *Methods*: RPMI 2650 (human nasal carcinoma), paclitaxel (Tx) and melphalan (Ml)-resistant cell lines were included in this study (3). Flasks of these cells were set up in duplicate at a confluency of approximately 50-60%. Aliquots of the conditioned media were taken at intervals of 24 hours over a 96-hour period, passed through a 0.22μm filter and were subsequently stored at -80˚C. Total RNA was extracted from 100μl of the conditioned media using the SV Total RNA Isolation system (Promega). RNA was also extracted from the corresponding cultured cells to be used as positive controls for all RT-PCR experiments. The presence and integrity of the RNA in all samples was confirmed by RT-PCR amplification of β-actin. *Results*: The message for β- actin was present in all of the conditioned media samples. CK-19 expression was also present in the majority of samples (approximately 70%). Other gene transcripts detected were HnRNP B1 and MDR1. The repeatability of these RT-PCRs however was unreliable. *Conclusion*: The results from this study indicate that gene transcripts are detectable in cell-free medium used to grow cancer cells, but not in control medium not exposed to cells by tumour cells. However, the presence of tumour- related mRNAs in these samples is apparently quite low, possibly near the limit of detection by RT-PCR. Alternative ways of concentrating the samples to improve the rate of repeatability of the RT-PCRs are being investigated.

References
1 Chen XQ, Bonnefoi H, Pelte MF, Lyautey J, Lederrey C, Movarekhi S, Shaeffer P, Mulcahy HE, Meyer P, Stroun M and Anker, P: Telomerase RNA as a detection marker in the serum of breast cancer patients. Clin Cancer Res *6*: 3823-3826, 2000.
2 Kopreski MS, Benko FA, Kwak LW and Gocke CD: Detection of tumour messenger RNA in the serum of patients with malignant melanoma. Clin Cancer Res *5*: 1961-1965, 1999.
3 Liang Y, Meleady P, Cleary I, McDonnell S, Connolly L and Clynes M: Selection with melphalan or paclitaxel (Taxol) yields variants with different patterns of multidrug resistance, integrin expression and *in vitro* invasiveness. Eur J Cancer *37*: 1041-1052, 2001.

*Acknowledgement*s
This work was supported by funding from the Higher Educational Authority's PRTLI programme, Ireland.

CHARACTERIZATION OF p53 WILD-TYPE AND NULL ISOGENIC COLORECTAL CANCER CELL LINES RESISTANT TO 5-FLUOROURACIL, IRINOTECAN OR OXALIPLATIN

John Boyer, Estelle G. McLean, Somaiah Aroori, Peter Wilson, Daniel B. Longley and Patrick G. Johnston

Cancer Research Centre, Queen's University, Belfast, N. Ireland. E-mail: e.mclean@qub.ac.uk

Aims: To generate and fully characterize a panel of 5-Fluorouracil (5-FU), Irinotecan (CPT-11) and oxaliplatin-resistant p53 wild-type and null isogenic cell lines derived from HCT116 colorectal carcinoma cells and to use these model systems to facilitate the identification of novel predictive markers to 5-FU, CPT-11 and oxaliplatin-based chemotherapy. *Methods*: A panel of 5-FU, CPT-11 and

oxaliplatin-resistant p53 wild-type and null isogenic sublines were derived from HCT116 colorectal carcinoma cells by repeated exposure to stepwise increasing concentrations of each chemotherapy over a period of ten months. Cytotoxicity studies were performed using cell viability and clonogenic cell survival assays to determine IC_{50} values for each chemotherapy in parental and resistant cell lines. Cell cycle analyses were carried out using a Beckman Coulter Epics XL Flow Cytometer. Immunoblotting was used to study PARP cleavage in both untreated and treated, parental and resistant cell lines. mRNA expression of genes implicated in 5-FU, oxaliplatin and CPT-11 resistance was determined by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. *Results*: We have successfully generated a panel of isogenic p53 wild-type and null HCT116 colorectal cancer cell lines resistant to 5-FU, oxaliplatin or CPT-11. In the p53 wild-type setting, IC_{50}(72h) values for 5-FU, oxaliplatin and CPT-11 were increased 3.0-, 31.0- and 10.0- fold in their respective resistant lines compared to parental cells. (Table I).

Table I. *IC_{50} values for 5-FU, oxaliplatin and CPT-11 in parental cell lines and their respective resistant sublines.*

	IC_{50} (μM)			
	p53 wild-type		p53 null	
Drug	Sensitive	Resistant	Sensitive	Resistant
5-FU	4.3	12.7	19.7	178.2
Oxaliplatin	0.3	9.4	1.7	17.9
CPT-11	3.2	30.3	11.4	200.4

In the p53 null setting IC_{50}(72h) values for 5-FU, oxaliplatin and CPT-11 were increased 9.0-, 10.5- and 17.5-fold in their respective resistant lines compared to parental cells. Using flow cytometric analysis, we demonstrated compromised apoptosis and G2 arrest in oxaliplatin and CPT-11 resistant cells, and compromised apoptosis and G1/S- phase arrest in 5-FU-resistant cells relative to the respective parental lines. Western blot analysis demonstrated reduced levels of apoptosis in 5-FU, oxaliplatin and CPT-11-resistant cells compared to parental cells following treatment with an IC_{50}(72h) dose of the appropriate drug for 48 h. In addition, cytotoxicity studies, cell cycle and Western blot analyses demonstrated decreased sensitivity to 5-FU and oxaliplatin, but not CPT-11, in p53 null cells compared to p53 wild-type cells. These data suggest a role for p53 as a predictor of response to 5-FU and oxaliplatin, but not CPT-11. RT-PCR analyses demonstrated modulation of genes previously implicated in 5-FU, oxaliplatin and CPT-11 resistance in p53 wild-type and null drug-resistant cells compared to parental cells. *Conclusion*: These model systems are currently being used as tools in conjunction with cDNA microarray and proteomic technologies to identify novel predictive markers to 5-FU, oxaliplatin and CPT-11-based chemotherapy in both p53 wild-type and null settings. The ultimate goal of this research is to identify a panel of markers that predict whether a tumour will respond to a particular chemotherapy and thus to tailor treatment regimens to the molecular profile of the tumour and patient. Such an approach has the potential to dramatically improve response rates and would spare patients the harmful side-effects of chemotherapeutic treatments from which they are unlikely to benefit.

A PARADIGM APPROACH FOR HIGH-THROUGHPUT FUNCTIONAL VALIDATION OF CANDIDATE TUMOUR-ASSOCIATED GENE FAMILIES: PROTEIN TYROSINE PHOSPHATASES AS A CASE STUDY

Gemma A. Cannon[1], Stephen E. Donoghue[2], David J. Mackin[1], David J. Easty[2] and William M. Gallagher[1]

Departments of [1]Pharmacology and [2]Pathology, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland. E-mail: William.Gallagher@ucd.ie

With the advent of high-throughput DNA sequencing and microarray technologies, a wealth of candidate tumour-associated genes have been provisionally identified. However, a current limitation now exists with respect to functional validation of putative mediators of tumour development and progression. Protein tyrosine phosphatases (PTPs) have been implicated in a variety of cellular processes, including the inhibition of cell growth and the suppression of tumourigenicity. However, the exact nature of how PTPs function has yet to be determined. Herein, we describe a multiplatform approach, employing high-throughput *in silico* and *in vitro* analysis *via* advanced bioinformatic and automated robotic techniques, respectively, to generate a comprehensive PTP cDNA resource. Survey of the UniGene database (www.ncbi.nlm.nih.gov/UniGene) revealed 234 unique clusters containing the term 'phosphatase'. Seventy-five of these phosphatase clusters were selected on the basis of having a characteristic PTP family protein signature, namely the CX_5R active site motif. Representatives from the following four PTP subfamilies were found: cytoplasmic, receptor-like, dual-specificity and low molecular weight PTPs. Hierarchical cluster analysis of the 75 PTP cDNAs on the basis of sequence homology *via* ClustalX (ftp://ftp-igemc.u-starseg.fr/pub/ClustalX/) mostly confirmed the separation of these subfamilies. The Mammalian Gene Collection (MGC)

was searched for full-length PTP cDNA clones, with 43 of the 75 denominated PTPs being obtained from this resource; however, 13 of these were disregarded following DNA sequencing. For those transcripts for which an MGC source was unavailable, long-range PCR amplification and recombinant cloning was undertaken (20 PTP cDNAs have been isolated in this manner so far). Automated plasmid purification of all independent PTP cDNA clones (50 in total) was carried out using the BioRobot 3000 (Qiagen), together with several Turbo 8 plasmid purification kits (Qiagen). Plasmids were examined qualitatively and quantitatively by gel electrophoresis and spectrophotometry, respectively. Inserts were amplified by PCR using vector-specific primers and subsequently automatically purified using a QIAquick 96 PCR purification kit (Qiagen). Greater than 95% recovery of PCR products was observed. These PCR products are currently being used to create a focused cDNA microarray centred around the PTP family. This PTP-specific cDNA microarray will ultimately have use in examining the expression of PTPs in melanoma cell lines and biopsies. The PTP cDNA resource is also being used to create mammalian cell expression constructs, *via* the Gateway cloning system, for use in downstream functional analyses; in particular, cell-based microarrays.

Acknowledgements
This work is funded by Cancer Research Ireland.

EXPRESSION LEVELS OF FIBULIN-1 AND –4 MRNA IN COLON CANCER TISSUES DETERMINED BY REAL-TIME QUANTITATIVE REVERSE TRANSCRIPTASE-PCR

Ilse-Maria Nolan[1], Edward JP. Fox[2], Catherine Moss[3], Dermot T. Leahy[2], Richard Geraghty[4], Hugh E. Mulcahy[4], John P. M. Hyland[4], David Fennelly[4], Kieran Sheahan[4], Diarmuid O' Donoghue[4], W. Scott Argraves[5] and William M. Gallagher[1]

[1]Department of Pharmacology, [2]Department of Pathology and [3]Core Technology Group, Conway Institute of Biomolecular and Biomedical Research, University College Dublin; [4]Centre for Colorectal Disease, St. Vincent's University Hospital, Dublin, Ireland; [5]Department of Cell Biology and Anatomy, Medical University of South Carolina, U.S.A. E-mail: ilsenolan@yahoo.com", william.gallagher@ucd.ie

Background: The fibulins represent a family of extracellular matrix proteins characterised by a unique C-terminal fibulin-type module and several repeated epidermal growth factor-like domains. The role of fibulin-1 in cancer remains controversial, while studies from this group have demonstrated a possible oncogenic role for fibulin-4. In more detail, we have previously shown dysregulated expression of fibulin-4 mRNA in a discrete series of paired colon tumour/normal tissues. Here, we present an original real-time quantitative reverse-transcriptase-PCR (RT-qPCR) method for detection of fibulin-1 and –4 mRNA expression. This RT-qPCR assay utilizes a dual-labelled fluorogenic probe and TaqMan technology for real-time assessment of PCR amplification. By this method, we have measured fibulin-1 and fibulin-4 mRNA expression in a wide range of paired colon tumour and normal tissue biopsies, along with a series of tumour-derived cell lines. *Methods*: Total RNA was extracted from powdered frozen tissue, integrity assessed and single-stranded cDNA prepared from 1μg total RNA. Primers and fluorescently-labelled probes were designed using Primer Express software. The following controls were used: (a) plus/minus reverse transcriptase (to determine level of genomic DNA contamination), (b) full-length fibulin-1 and –4 cDNA–containing plasmids (positive controls), (c) no template (negative control) and (d) 18S rRNA as a 'housekeeping control'. Data analysis was carried out by the standard curve method of quantitation. *Results*: We used serial dilutions (copy number range from 10^1 to 10^9) of fibulin-containing plasmids to assess the increased sensitivity of these real-time PCR assays over conventional PCR. The real-time PCR assay was found to be sensitive down to 10 copies, whereas conventional PCR was sensitive down to 10^7 copies. Fibulin-4 mRNA expression was assessed in a panel of human tumour-derived cell lines, including two breast tumour cell lines, one ER-positive (T47-D) and the other ER-negative (MDA-MB-231). A 10-fold elevation of fibulin-4 mRNA expression in the ER-positive cell line was observed, possibly suggestive of a link between fibulin-4 expression and ER status. Assessment of fibulin-4 mRNA expression in a three-membered isogenic series of increasingly tumourigenic melanoma cell lines (WM793, WM793-P1 and WM793-P2) showed a two-fold increase in the third and most tumourigenic derivative of the family (WM793-P2), compared to the other two cell lines. In an extensive panel of paired colon tumour and normal tissue biopsies (n = 27 pairs), we observed elevated fibulin-4 mRNA expression in tumour *versus* normal tissues (p value <0.0001, non-parametric matched Wilcoxan test). In a smaller number of paired colon tumour/normal tissues (n = 22), we noticed a partial trend towards up-regulated fibulin-1 expression in tumour *versus* normal biopsies (p value = 0.1075, matched Wilcoxon test). *Conclusion*: This study describes a novel means of quantifying fibulin-1 and -4 mRNA expression. The assays have been thoroughly evaluated using stringent controls, as well as various tumour-derived cell line model systems. We also report our findings on fibulin expression patterns between paired normal and tumour tissues in the colon. Our findings support previous work in our lab regarding fibulin-4 expression in tumour material and confirm a possible involvement of fibulin-4 overexpression in the pathogenesis of colon cancer.

Acknowledgements
Financial support is acknowledged from Enterprise Ireland, the Association for International Cancer Research and the Health Research Board, Ireland.

MODULATION OF CYTOKERATIN EXPRESSION IN POORLY-DIFFERENTIATED LUNG CARCINOMA CELL LINES, DLKP AND A549, FOLLOWING EXPOSURE TO URIDINE ANALOGUES

Jason McMorrow, Paula Meleady and Martin Clynes

National Institute of Cellular Biotechnology, Dublin City University, Dublin 9, Ireland. E-mail: Jason.mcMorrow4@mail.dcu.ie

Bromodeoxyuridine (BrdU) is a thymidine analogue capable of inducing epitheloid morphology and altering the expression of neuroendocrine markers in Small Cell Line Carcinoma (SCLC) cell lines. The ability of BrdU to alter differentiation in neuronal, muscle and haematopoietic lineages has been well documented in the literature. Following on from work previously carried out in our laboratory, the BrdU analogues Chlorodeoxyuridine (CdU) and Iododeoxyuridine (IdU) were studied to investigate if they possessed similar differentiating properties to BrdU. These two analogues, like BrdU, are incorporated into DNA instead of thymidine. Evidence suggests that this incorporation into the DNA alters the DNA's conformation, which in turn may affect interactions with specific transcription factors, leading to either inhibition or induction of differentiation.

The DLKP cell line was established in our laboratory from a tumour histologically diagnosed as a poorly-differentiated lung carcinoma. DLKP cells have properties which suggest they could be classified as either SLCL-V (variant) or non-small cell lung carcinoma with neuroendocrine differentiation (NSCLC-NE). DLKP contains neurofilament and vimentin proteins, but no keratin proteins or other epithelial markers such as epithelial membrane antigen, EP16 or desmosomal proteins are detectable by cytochemistry. We have shown (at protein level) that the DLKP and A549 cell lines upon treatment with CdU and IdU showed increased expression of the integrin subunits α_2 and β_1, as well as the cellular adhesion molecule, Ep-CAM and of cytokeratins 8, 18 and 19.

2-DEOXYGLUCOSE AS A POSSIBLE ANTI-METABOLITE IN DRUG-RESISTANT VARIANTS OF DLKP, A HUMAN LUNG CANCER: INITIAL STUDIES

Joanne Keenan and Martin Clynes

National Institute for Cellular Biotechnology, Dublin City University, Dublin 9, Ireland. E-mail: Joanne.Keenan@dcu.ie

The accumulation of 2-deoxyglucose (2-DG), a glycolytic inhibitor, was investigated in a human lung carcinoma DLKP and drug-resistant variants to assess if the glycolytic potential of these cells may offer a selective means of reducing resistance. For the highly resistant variants DLKP A and A5F, 2-DG uptake was increased while for A2B, A10, C14 and A25010p, variants with varying resistance levels, lower uptake was observed. These studies reveal the relationship between 2-DG uptake and drug resistance to be complex for DLKP and variants with no one factor being responsible and showed no consistent trend between cancer and drug-resistant variants, limiting the use of glycolytic inhibitors to reduce resistance in variants of DLKP.

ENDOTHELIUM AS A TARGET IN THE DESIGN OF NOVEL TUMOUR VASCULATURE TARGETING AGENTS

John J. Walsh, E.M. McCormack and R. Shah

Department of Pharmacognosy, School of Pharmacy, Trinity College Dublin, Ireland. E-mail: jjwalsh@tcd.ie

Targeting tumour angiogenesis/vasculature has a number of distinct advantages over direct tumour cell targeting as; (i) there are significant morphological, anatomical and behavioural differences between normal and tumour blood vessels, (ii) tumour blood vessels are essentially composed of endothelial cells and are directly accessible to intravenously injected agents, (iii) only a minority of endothelial cells within a capillary need to be killed to cause complete closure of the vessel, (iv) amplification of cell death occurs as a large number of cancer cells die after the destruction of one vessel, (v) endothelial cells are genetically stable, (vi) a single angiogenic-targeting agent should be suitable for targeting most or all types of solid tumours and (vii) the vasculature in the adult is normally quiescent, with the exception of highly ordered processes such as in the female reproductive cycle.

Based on the recent published data on the functional role of aminopeptidase N (1,2) and the inhibitory effects of tubulin binding agents (3,4) in angiogenesis/vasculature, a clearly novel and innovative approach is to design molecules that have the potential to prevent vascularisation by inhibition of both endothelial cell proliferation and morphogenesis. Our approach was to engineer a innovative molecular design incorporating into the initial design components of known tubulin and aminopeptidase N inhibitors. As bestatin, a known aminopeptidase N inhibitor, is in clinical use and can be tolerated at exceptionally high doses without apparent side-effects, it was postulated that the ultimate molecular design

should incorporate this molecule as part of the overall structure. Colchicine was chosen as the model tubulin inhibitor. Based on this observation, we synthesised a series of peptides possessing in addition to aspartic acid, the amino acid, (2S,3R)-3-amino-2-hydroxy-4-phenylbutanoic acid, which is the key amino acid required for inhibition of aminopeptidase N. The attachment of these peptides to N-deacetylcolchicine proceeded smoothly, using standard peptide coupling methodology. Significantly all of the compounds synthesised are more potent than bestatin in the aminopeptidase N assay (5). The tubulin binding data obtained on these compounds clearly indicates that, for potent dual inhibition, the maximum size of the peptide should not exceed three amino acids and, in addition, the third amino acid should not possess a bulky side chain.

References

1 Pasqualini R, Koivunen E, Kain R, Lahdenranta J, Sakamoto M, Stryhn A, Ashmun RA, Shapiro LH, Arap W and Ruoslahti E: Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. Cancer Res *60*: 722-727, 2000.

2 Bhagwat SV, Lahdenranta J, Giordano R, Arap W, Pasqualini R and Shapiro LH: CD13/APN is activated by angiogenic signals and is essential for capillary tube formation. Blood *1*: 652-659, 2001.

3 Dark GG, Hill SA, Prise VE, Tozer GM, Pettit GR and Chaplin DJ: Combretastatin A-4, an agent that displays potent and selective toxicity toward tumour vasculature. Cancer Res *57*: 1829-1834, 1997.

4 Kanthou C and Tozer GM: The tumor vascular targeting agent combretastatin A-4-phosphate induces reorganization of the actin cytoskeleton and early membrane blebbing in human endothelial cells. Blood *99*: 2060-2069, 2002.

5 Melzig MF and Bormann H: Betulinic acid inhibits aminopeptidase N activity. Planta Med, 1998.

SHEAR-INDUCED ACTIVATION OF METASTATIC CELLS IS ENHANCED BY THE PLATELET SECRETEOME AND INHIBITED BY ASPIRIN.

Karen Lawler[1], Gerardene Meade[1], Gerald O' Sullivan[2] and Dermot Kenny[2]

[1]Department of Clinical Pharmacology, The Royal College of Surgeons, 123 St. Stephens Green, Dublin 2; [2]Cork Cancer Research Centre, Mercy University Hospital, University College Cork, Ireland. E-mail: Klawler@rcsi.ie

Platelets play a critical role in the dissemination of tumours through their interaction with metastatic cells. The anti-platelet agent aspirin has been shown to have an effect on cancer spread by mechanisms that are not fully understood. Recent evidence suggests that shear forces are important for both activation of receptors on platelets and cancer cells. *Aim*: We investigated the effect of the platelet secreteome in the presence and absence of aspirin on shear-induced activation of a novel oesophageal metastatic cell line. *Methods*: Oesophageal metastatic cells (RBM-3) were isolated from the rib bone marrow of a patient and cultured. Shear-induced activation of the metastatic cells was assayed using a modified parallel platelet flow chamber. Endothelial cells (ECs) were cultured in 35-mm dishes and stimulated with lipopolysaccharide. The ECs were then placed in chambers for the flow assay. Oeophageal metastatic cells were injected into the flow system and allowed to settle on the ECs for 5 minutes. The tumour cells were perfused over activated ECs at incremental venous shear rates from $50s^{-1}$ to $800s^{-1}$. In separate experiments, metastatic cells were preincubated with the platelet secreteome (aspirin or non-aspirin treated) and assayed under the same shear conditions. *Results*: Under flow conditions adherent metastatic cells remained quiescent until a shear rate of $400s^{-1}$ was reached. (n = 10). At this venous shear rate of $400s^{-1}$, the oesophageal cells rapidly formed blebs and pseudopods, which we termed shear-induced activation. In the presence of the platelet secreteome, the tumour cells became 'active' at a significantly lower shear rate of $50s^{-1}$. (n = 6). Thus the platelet secreteome potentiates activation of these cells under flow conditions. In the presence of aspirin-treated platelet secreteome, the potentiation of shear-induced activation was negated and the metastatic cells activate at $400s^{-1}$. (n = 6). *Conclusion*: The platelet secreteome can facilitate the invasive behaviour of this metastatic cell line, which is inhibited by pharmacologically relevant doses of aspirin.

EFFECTS OF EPIRUBICIN-SULINDAC COMBINATION THERAPY ON THE LUNG CANCER CELL LINE DLKP

Kieran Duffy, Robert O' Connor and Martin Clynes

National Institute for Cellular Biotechnology, Dublin City University, Dublin 9, Ireland. E-mail: Kieran.duffy2@mail.dcu.ie

It has previously been reported by our group that combining a number of specific non-steroidal antiinflammatory drugs (NSAIDs) with a variety of chemotherapeutic drugs results in a significant increase in the toxicity of the anti-cancer drug. This is due to inhibition of the MRP-1 cancer resistance mechanism. The NSAID sulindac is currently the subject of a clinical trial as a combination therapy with the anthracycline drug, epirubicin. In order to assess the potential implications of such a therapy, we have examined

the effect of long-term exposure of a lung cancer cell line (DLKP) to both epirubicin and sulindac. DLKP cells were exposed to epirubicin alone (0.2μM) and epirubicin in combination with 20μM sulindac for 4 hours on a weekly basis over a 6-week period. Analysis of the resistant variants indicated that co-selection with sulindac and epirubicin resulted in P-gp and MRP induction at similar levels to that of epirubicin-selected cells. The cross- resistance profiles of the selected cells were similar in the case of single selection and co-selection. HPLC quantification of the epirubicin efflux in the resistant variants indicated that epirubicin was effluxed to a much greater extent from the drug-treated cells than that of the parent DLKP cells, which indicated the emergence of efflux pumps in all selected cells. Western blot analysis confirmed this by demonstrating the induction of MRP-1 and P-gp after the first initial exposure to the drug.

THE INVOLVEMENT OF P53 ON RESISTANCE TO CHEMOTHERAPY IN LUNG CANCER CELLS

Laura Breen, Verena Amberger-Murphy, Deirdre Cronin, Mary Heenan and Martin Clynes

National Institute for Cellular Biotechnology, Dublin City University, Dublin 9, Ireland. E-mail: laura.breen3@mail.dcu.ie

p53 is a tumour suppressor gene, which is mutated in more than 50% of all tumours. Most chemotherapeutic drugs cause DNA damage that is sensed by p53, which either repairs the damage or induces apoptosis. In case of a mutation or deletion in the p53 gene, the effectiveness of the chemotherapy is compromised. The aim of this study was to investigate the sensitivity to chemotherapy drugs in a range of cell lines with different p53 status which had been exposed to sequential pulses of drugs. The cell lines used were A549 (wild-type p53), NCI-H1299 (p53 null) and DLKP-SQ (mutant p53). These cells were treated with a high kill concentration of taxol or carboplatin once a week for ten weeks, resulting in resistant populations of cells. The resulting cell lines were tested for sensitivity to the following drugs – taxol, adriamycin, VP-16, carboplatin, cisplatin and 5-fluorouracil. These profiles were compared to those of the parent cell lines. After pulse selection the p53 status of the A549 cells, which express wild-type p53, was assessed by immunohistochemistry and Western blotting.

FIBULIN-1 VARIANT-SPECIFIC EFFECTS ON CELLULAR MIGRATION AND GLOBAL PROTEIN EXPRESSION IN HT1080 FIBROSARCOMA CELLS

Lisa M. Greene[1], Benjamin Reed[2], Christian Knaak[3], W. Scott Argraves[3] and William M. Gallagher[1]

[1]Department of Pharmacology, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Dublin 4, Ireland; [2]Ciphergen Laboratories, Guildford, Surrey, U.K.; [3]Department of Cell Biology and Anatomy, Medical University of South Carolina, 173 Ashley Avenue, Charleston, South Carolina, U.S.A. E-mail: greenelisa@hotmail.com

The fibulins are a novel family of secreted glycoproteins characterised by repeated epidermal growth factor-like domains and a unique C-terminal structure. Four splice isoforms of fibulin-1 have been identified to date, designated A-D. There is accumulating evidence to suggest that fibulin-1 may play an active role in tumourigenesis. For example, fibulin-1D can suppress *in vitro* invasion of a number of human tumour-derived cells, as well as extend the latency of fibrosarcoma tumour growth *in vivo*. In addition, we have shown that fibulin-1 protein levels are elevated in breast (1) and colon cancers (n=20; $p=0.02$;) (2). Furthermore, a recent study has shown that the ratio of fibulin-1C:-1D mRNA was increased in ovarian carcinomas (3). Additionally, fibulin-1C and –1D splice variants exhibit different binding affinities for various extracellular matrix proteins. Taken together, these findings suggest that fibulin-1 splice variants may display different functional activities. To date, however, functional studies on the fibulin-1C splice variant are limited. In this study, therefore, we sought to detect fibulin-1 variant-specific effects on cellular migration and global protein expression in HT1080 fibrosarcoma cells. Interestingly, overexpression of fibulin-1C, but not fibulin-1D, increased the migration of HT1080 cells towards fibronectin (n=3; $p=0.03$). Using ProteinChip (Ciphergen) technology, we identified a number of differentially expressed proteins in the fibulin-1 transfected cells as compared to control cells. The mass spectrometry profiles obtained in this study will greatly accelerate the identification of proteins involved in fibulin-1-dependent signalling pathways. Overall, the results presented demonstrate a functional diversity in fibulin-1 mediated by alternative splicing. Moreover, the balance of fibulin-1C and –1D splice variants may have important implications in tumour development and progression.

References

1 Greene LM *et al.*: Br J Cancer *88*: 871-878, 2003.
2 Greene LM: Ph.D. Thesis. National University of Ireland, Dublin, 2003.
3 Moll F *et al.*: Oncogene *21*: 1097-1107, 2002.

Acknowledgements

Funding for this work is acknowledged from the Irish Cancer Society, Enterprise Ireland, the Association for International Cancer Research and the European Commission.

INVESTIGATION OF DRUG-RESISTANT CELLS USING PROTEOMIC TECHNOLOGIES

Lisa Murphy, Joanne Keenan, Paula Meleady and Martin Clynes

National Institute for Cellular Biotechnology, Dublin City University, Dublin 9, Ireland. E-mail: Lisa.Murphy@mail.dcu.ie

Proteomics can be viewed as the systematic analysis of a set of proteins for their identification, quantification and determination of functionality. Proteome analysis is most commonly accomplished by the combination of two-dimensional gel electrophoresis (2DE) and mass spectrometry (MS). Currently one of the most popular applications of proteomics is the area of cancer research. Early detection of cancer is crucial for a favourable prognosis, but classical tumour markers (proteins secreted by tumour cells) suffer from lack of specificity and sensitivity. Their prognosis potential is further hindered by the late stage of disease progression at which they present. Proteomics provides a means of identifying markers at an early stage of tumour development, with greater sensitivity and specificity for disease prognosis. In this project, 2DE is being optimised in order to investigate protein expression in a lung cancer cell line (DLKP) and a drug-resistant variant (DLKP-Mitoxanthrone). Proteins differentially expressed may have diagnostic value in relation to drug resistance assessment.

IDENTIFICATION OF THE ANTIGEN RECOGNISED BY THE MONOCLONAL ANTIBODY DESIGNATED 1A2

Michael Henry, John Milne, Lisa Connolly and Martin Clynes

National Institute for Cellular Biotechnology, Dublin City University, Dublin, Ireland. E-mail: Michael.henry@dcu.ie

The multidrug resistance protein 1 (MRP1) gene encodes a transporter protein that helps to protect cells against xenobiotics. Elevated levels of MRP1 in tumour cells can result in the active extrusion of a wide range of anticancer drugs. MRP1 encodes a 190KDa membrane glycoprotein. Antibody 1A2 was generated using a synthetic peptide corresponding to amino acids 1520-1531 from the N-terminal region of MRP 1. Using Western blot analysis, MAb 1A2 recognises a 190kD band on some multidrug-resistant (MRP1 overexpressing) cell lines but does not recognise a band on other multidrug-resistant (MRP1 overexpressing) cell lines. The epitope recognised by this antibody is heat stable. In an attempt to purify the antigen recognised by MAb 1A2, immuno-precipitation methods were investigated. However a selection of protein beads, sample preparation methods and incubation conditions all proved unsuccessful as a means of antigen purification. Chromatography, as an alternative to immunprecipitation, was used as a means of purification of the antigen recognised by 1A2. The starting material (positive cell line) was semi-purified using a 1ml QAE sepharose column (a strong anion exchanger). We have determined the salt concentration needed to elute the antigen of interest from the column. Further chromatography using gel filtration and hydrophobic interaction chromatography will allow further purification of the starting material. We have successfully bound MAb 1A2 to 1ml protein G columns, which represents the basis for affinity chromatography using the semi-purified starting material from the QAE sepharose chromatography containing the antigen specifically recognised by the MAb 1A2.

Chromatography should facilitate the purification of this antigen and allow for its identification by Mass Spectrometry.

IMMUNODETECTION OF ORF1 PROTEIN ENCODED BY THE L1 RETROTRANSPOSON IN HUMAN CELL LINES

Olga Piskareva and Vadim Schmatchenko

Laboratory of Mobile Genetic Elements, Institute of Biochemistry and Physiology of Microorganisms, RAS, Pushchino, 142290, Russia. E-mail: olpi@ibpm.pushchino.ru

LINE-1 or L1 elements belong to an extremely abundant class of non-LTR retrotransposons and comprise approximately 17% of human genomic DNA. The consensus L1 elements are about 6-7 kb long and contain both 5' and 3' UTR, and two non-overlapping open reading frames (ORFs). ORF1 encodes a 40 kDa nucleic acid binding protein associated with L1 RNA in cytoplasm. It has been shown in previous studies that ORF1 protein is expressed in some human cell lines and tumors.

In this study, we analyzed expression of the L1 ORF1 protein in various human cell lines. The ORF1 protein was expressed as fusion of glutathione-S-transferase in *E.coli* and purified from bacterial lysates by affinity chromatography. The purified fusion protein was used to raise polyclonal mouse and rabbit anti-ORF1 antibodies. By Western blot analysis, we examined a number of human cell lines. The ORF1 protein was found in varying amounts in the cell lines tested. No immunoreactive band was observed in normal human blood lymphocytes. The L1 ORF1 protein is likely to be produced in very low amounts in some cancer cells, therefore reproducible results have been difficult to obtain with both affinity purified and total polyclonal antibodies. We conclude that using monoclonal antibodies against the L1 ORF1 protein improves the immunodetection of this protein in cancer cells.

THE TRANSCRIPTION FACTORS GATA-2 AND GATA-3 MAY PLAY A ROLE IN REGULATING THE BRDU-INDUCED EXPRESSION OF VARIOUS MULTIDRUG TRANSPORTERS IN LUNG CANCER CELLS

Padraig Doolan, Niall Barron, Yizheng Liang and Martin Clynes

National Institute for Cellular Biotechnology, Dublin City University, Dublin, Ireland. E-mail: padraig.doolan@dcu.ie

Previous studies in this laboratory have demonstrated that exposure of lung epithelial cancer cell lines to the differentiating agent BrdU resulted in increased expression of various proteins not normally expressed in those lines. In this study, RT-PCR analysis of DLKP cells exposed to BrdU demonstrated increased gene expression of the multidrug resistance-associated protein MRP1 and two of its analogues, MRP2 (cMOAT) and MRP3 (MOAT-D). Nuclear Run-On assays using probes for the MRP1, MRP2 and MRP3 genes confirmed that the increase in expression was due to increased transcription initiation rates of these genes. Western blotting analysis of the BrdU-treated DLKP also revealed increased MRP1 protein expression. *Transfac*™-mediated transcription factor analysis of the 5 promoter regions of nine genes up-regulated by BrdU identified seven potentially shared transcription factors. RT-PCR analysis using primers for the seven identified factors revealed increased expression of the zinc-finger binding domain factors GATA-2 and GATA-3 in DLKP cells exposed to BrdU. These results demonstrate that the transcription factors GATA-2 and GATA-3 may be involved in BrdU-mediated up-regulation of expression of these genes during differentiation of DLKP cells.

SULINDAC AS A MODULATOR OF MRP-1 IN EPIRUBICIN CANCER THERAPY. VALIDATION OF A PROTOCOL FOR HPLC ANALYSIS OF PATIENT BLOOD SPECIMENS FROM A CANCER CLINICAL TRIAL

Paula Kinsella[1], Robert O'Connor[1], Michael O'Leary[2] and Martin Clynes[1]

[1]National Institute for Cellular Biotechnology, DCU, Dublin 9; [2]Dept. of Medical Oncology, St Vincent's Hospital, Dublin 4, Ireland. E-mail: paula.kinsella@dcu.ie

A clinical trial involving a combination of an NSAID, sulindac and the chemotherapeutic drug, epirubicin, is in progress to assess the role of sulindac as a modulator of the resistance mechanism, MRP-1. Patient blood samples have been taken at intervals throughout the clinical trial. HPLC analysis will be used as a method for determining the exact quantity of both drugs in each patient's blood specimens. This poster reports validatory aspects of the two HPLC methods to be used. As epirubicin is a fluorescent drug, the HPLC analysis of epirubicin involved the use of fluorescence and a UV detector, whereas the HPLC analysis of sulindac involved the use of a UV detector. The respective methods were validated using standards of epirubicin and sulindac, which were made up with the addition of fetal calf serum. The analysis involved three sets of intra-day extraction standards, and three sets of inter-day extraction standards with internal standards of daunorubicin for epirubicin analysis and indomethacin for sulindac analysis. Results are reported indicating the suitability of these methods. Epirubicin gave a peak at 5.5 minutes and sulindac gave a peak at 1.5 minutes.

CLONING AND EXPRESSION OF A MAMMALIAN DIPEPTIDASE ENZYME FOR USE IN ANTIBODY-DIRECTED ENZYME PRODRUG THERAPY

Rafia Razzaque[1,2], Ronan O'Dwyer[1], Edmond Magner[1,2], Timothy P. Smyth[1] and J. Gerard Wall[1,2]

[1]Department of Chemical and Environmental Sciences and [2]Materials and Surface Science Institute, University of Limerick, Plassey Technological Park, Limerick, Ireland. E-mail: Rafie.Razzaque@ul.ie

Aims: To clone and express a human dipeptidase enzyme in recombinant form in *Escherichia coli* for use in antibody-directed enzyme prodrug therapy (ADEPT). *Methods*: The structural gene encoding the mammalian dipeptidase (MDP) enzyme (1) was amplified from human kidney cDNA. The cloning procedure introduced an *E. coli ompA* signal sequence and peptide tag at the N-terminal end of the gene and removed the membrane anchor from the C-terminal end. The recombinant gene was cloned into a periplasmic expression vector and produced in soluble form in *E. coli.* Protein production and activity were monitored by immunoblotting, determination of antibiotic resistance *in vivo*, and *in vitro* fluorimetric assays. *Results*: The human gene was cloned and sequenced in pCR4-TOPO (Invitrogen Corp.), followed by subcloning into the pIG6 periplasmic expression vector (2). SDS-PAGE and immunoblotting revealed the presence of a polypeptide of the correct length in the bacterial periplasm but screening on the carbapenem, imipenem, failed to identify clones with increased minimal inhibitory concentrations (MICs) for the antibiotic. Cell fractionation and *in vitro* fluorimetric analysis of periplasmic

fractions revealed higher dipeptidase activity in induced MDP-containing clones, with increasing activity observed with higher levels of added inducer. Ongoing efforts at increasing functional expression levels of the enzyme include co-expression of rare tRNA genes, and co-production of the disulfide bond isomerase, DsbC, in the *E. coli* expression host. *Conclusion*: Human renal dipeptidase has been cloned and produced in functional form in an *E. coli* host. Increased expression levels will be used to increase its usefulness as the enzyme moiety in ADEPT applications.

References

1 Smyth TP, Wall JG and Nitanai Y: A substrate variant as a high-affinity, reversible inhibitor: insight from the X-ray structure of cilastatin bound to membrane dipeptidase. Bioorg Med Chem *11*: 991-998, 2003.

2 Ge L, Knappik A, Pack P, Freund C and Plückthun A: Expressing antibodies in *Escherichia coli. In*: Antibody Engineering, 2nd ed. CAK Borrebaeck, ed. Oxford University Press, Oxford, pp. 229-266, 1995.

INVESTIGATION OF MULTIPLE DRUG RESISTANCE AND INVASION INDUCTION BY ANTICANCER DRUGS IN THE HUMAN LUNG CARCINOMA CELL LINE, DLKP.

Rasha Linehan, John Cahill, Irene Oglesby, Lorraine O'Driscoll, Yizheng Liang and Martin Clynes

National Institute for Cellular Biotechnology, Dublin City University, Dublin 9, Ireland. E-mail: rasha_ramzi@yahoo.com

DLKP, a human lung carcinoma cell line, with a low invasive phenotype, was pulse selected with three chemotherapeutic drugs, taxotere, 5-fluorouracil and vincristine. Two of the three resulting variants (DLKP-taxotere and DLKP-vincristine) exhibited a multiple drug resistance profile, while the profile of DLKP-5-fluorouracil was unchanged. Furthermore, DLKP-taxotere displayed a dramatic increase in *in vitro* invasiveness and motility. However, there was no increase in *in vitro* invasiveness in the other two variants. RT-PCR and Western blot analysis revealed highly elevated levels of MDR-1 mRNA and protein expression. The expression level of MRP-1 mRNA was unaltered, while MRP-1 protein was over-expressed in DLKP-taxotere. MRP-2 and MRP-5 mRNA expression was absent from all cell lines, while MRP-3 was expressed only in DLKP-5FU. MRP-4 mRNA was expressed at a low level, and only in DLKP-taxotere and DLKP-vincristine. E-cadherin was over-expressed in DLKP-taxotere at both the protein and mRNA level. The expression of the anti-apoptotic genes, galectin-3 and survivin was also analysed. While expression of galectin-3 and survivin mRNA was not dramatically changed, survivin protein expression was down-regulated in DLKP-vincristine and almost absent in DLKP-taxotere. The results indicate that gene expression changes occur at both transcriptional and translational levels. Gelatin zymography results showed that DLKP and its three variants secrete MMP-2, MMP-9 and MMP-13, with DLKP-5-fluorouracil secreting the most MMPs.

BIOASSAY OF ANTI-CANCER CONSTITUENTS OF *NIGELLA SATIVA*, WITH SPECIAL REFERENCE TO MODE OF ACTION

Sara Rooney and Michael F. Ryan

Zoology Dept, University College Dublin, Belfield, Dublin 4, Ireland. E-mail: sarar@netsoc.ucd.ie

Seed of *Nigella sativa,* black cumin, has been used for thousands of years as an anti-inflammatory, anti-bacterial, anti-fungal and anti-helimenthic agent. More recently its effects as an anti-tumour agent have been studied. The major established anti-cancer constituent of the seed is a pentacyclic triterpene saponin, alpha-hederin (1), thymoquinone and dithymoquinone are also active (2). This project aims to investigate the efficacy of alpha-hederin against a range of cancer cell lines. Its mode of action and efficacy in comparison to and in conjunction with thymoquinone will also be established.The effect of alpha-hederin and thymoquinone on four human cancer cell lines namely lung carcinoma (A549), epidermoid larynx carcinoma (HEp-2), colon carcinoma (HT-29) and pancreatic carcinoma (MIA PaCa-2), were investigated, using the following assays: MTT-cell proliferation assay (3) to evaluate cell population density in response to drug addition; fluorescence microscopy using ethidium bromide and acridine orange to examine apoptosis/ necrosis (4); lactate dehydrogenase (zinc metalloenzyme) activity assay (5) to determine cell membrane damage upon drug addition. Alterations in cellular morphology in the four cell lines were assessed using phase contrast light microscopy. MTT assay absorbance values (replicates of 8) were analysed using SAS 8.0 as a one-way classification and IC_{50} values (inhibition concentration rate at 50%) were determined by linear regression. IC_{50} values (μM) for alpha-hederin respectively after 24, 48, 72h exposure on the four cell lines were as follows: A549: 10.97, 8.2, 9.86; HEp-2: 13.91, 13.4, 10.9, HT-29: 16.66, 14.6 12.5; MIA: 28.59, 24.9, 24.63μM. Thymoquinone was only cytotoxic to the MIA cell line, with IC_{50} values after 24, 48, 72h, of 63.32, 67.33 and 59.97μM.

Initial morphological examination of the four cell lines using fluorescence microscopy indicated alpha hederin induces apoptosis and necrosis. This will be quantified using Hoechst staining. Upon alpha-hederin/thymoquinone exposure, lactate dehydrogenase levels appeared unchanged, compared to

untreated control cells, suggesting no cell membrane damage had taken place. In all four cells lines, treatment with alpha-hederin altered cellular morphology: cells separated, decreased in number and became rounder with increased detachment from the substratum with increasing drug concentrations. Alpha-hederin is significantly cytotoxic to all cell lines with various efficacies. It appears to have no effect on the cell membrane, and initial results indicate that it does induce apoptosis. Thymoquinone is cytotoxic (2.4-fold less cytotoxic than alpha-hederin) to the pancreas cancer cell line and appears to have no effect on the cell membrane.

References

1 Swamy SMK and Huat BTK: Extraction, isolation and characterisation of anti-tumour principle, α-hederin, from the seeds of *Nigella sativa*. Planta Med *67*: 29-32, 2001.
2 Worthen DR, Ghosheh OA and Crooks PA: The *in vitro*, anti-tumour, activity of some crude and purified components of the black seed *N.sativa*. Anticancer Res *18*: 1527-1532, 1998.
3 Freshney IR: Culture of Animal Cells. Third ed.1994. Pub. Wiley-Liss.
4 Mc Gahon AJ, Martin SJ, Bissonette RP, Mahboubi A and Shi Y: The end of the cell line: Methods for the study of apoptosis *in vitro*. Methods Cell Biol *46*: 153-185, 1995.
5 Wroblewski F and Ladue J: Lactic dehydrogenase activity in blood. Proc Soc Exp Biol Med *90*: 210, 1955.

CONJUGATED LINOLEIC ACID ISOMERS INHIBIT BUTYRATE-INDUCED ALKALINE PHOSPHATASE ACTIVITY IN THE HT-29 HUMAN ADENOCARCINOMA CELL LINE.

S. Rahman[1], C. Stanton[2] and R. Devery[3]

[1]School of Biotechnology, Dublin City University, Dublin 9; [2]Teagasc, Dairy Products Research Center, Moorepark, Fermoy, Co. Cork; [3]National Institute for Cellular Biotechnology, Dublin City University, Dublin 9, Ireland. E-mail: rosaleen.devery@dcu.ie

Background: A precise balance between cell renewal, cell differentiation and cell death maintains tissue homeostasis in the colon. Dietary lipids, in particular butyrate, are important factors stimulating differentiation of colon cells both *in vivo* and *in vitro*. Dietary conjugated linoleic acid (CLA), a group of positional and geometric isomers of linoleic acid, has been shown to reduce colon tumour incidence in rodents by mechanisms probably involving apoptosis. The HT-29 human colon adenocarcinoma cell line is a relevant model for studying the modulatory role of fatty acids on intestinal cell growth and differentiation. *Aims*: The objective of this study was to determine whether the effects of three commercial CLA preparations (pure *c*9*t*11-CLA, pure *t*10*c*12-CLA and a CLA mixture, containing 29.5% *c*9*t*11 and 29% *t*10*c*12-CLA) on HT-29 cell growth correlate with the effects on alkaline phosphatase (ALP), a specific brush border membrane enzyme used as a marker of HT-29 colon cell differentiation. *Methods*: Stock solutions of sodium salts of fatty acids (20mg/ml) were prepared in medium containing BSA (10mg/ml) as described by Ip *et al*. (Experimental Cell Research *250*: 22-34, 1999). HT29 cells were maintained in Dulbecco's Minimum Essential Medium supplemented with 5% (v/v) fetal calf serum, 1 unit/ml streptomyosin and penicillin, 0.2mM L-glutamine, 1mM HEPES and incubated at 37˚C in a humidified tissue culture incubator with 5% CO_2. Initially, cells were seeded at 0.8 x 10^6 cells/ ml in T75-cm^2 flask in culture media. Next day, the media was replenished with fresh media containing sodium butyrate (1-5mM) and CLA (50-125 μM). Control flasks were supplemented with media containing BSA (10μg/ml). Sodium butyrate (NaBt), a potent controller of cell growth and differentiation was used as a positive control. Cell growth was monitored by trypan blue exclusion. ALP activity was measured in cell lysates (containing up to 600 μg protein) by the kinetic determination of p-nitrophenol phosphate (15mM) hydrolysis using a commercially available Sigma diagnostics kit. *Results*: Both NaBt (1-5 mM) and CLA preparations (50-125 μM) decreased cell growth in a dose-dependent manner. After 3 days incubation with NaBt (3 mM), growth was inhibited by 55% relative to control. Co-incubation with the CLA mixture, *c*9*t*11-CLA and *t*10*c*12-CLA (all at 75 μM) reduced growth by 75%, 67% and 78%, respectively. Despite synergistic effects on growth, effects on intestinal differentiation in HT29 cells were discordant. NaBt (3 and 5 mM) induced significant ($p<0.05$) increases in ALP activity for up to 9 days relative to controls. Neither the CLA mixture, *c*9*t*11-CLA nor *t*10*c*12-CLA in the range 50-125 μM increased ALP activity. When used in combination, CLA preparations (75 μM) attenuated NaBt (3 mM)-induced ALP activity. *Conclusion*: These data suggest that growth inhibition induced by isomers of CLA may be mediated by a differentiation-independent pathway.

PREDICTING THE RESPONSE OF GYNAECOLOGICAL TUMOURS TO CHEMOTHERAPY

Sharon A.O'Toole, B.L. Sheppard, E.P.J. McGuinness, N.C. Gleeson and J. Bonnar

Department of Obstetrics and Gynaecology, Trinity Centre for Health Sciences, St. James's Hospital, Dublin 8, Ireland. E-mail: shotoole@tcd.ie

Aims: The aim of this study was to use an *in vitro* (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-

sulfophenyl)-2H-tetrazolium, inner salt) (MTS) assay to determine the response of ovarian, endometrial and cervical tumours to various cytotoxic drugs and correlate this with the *in vivo* response. *Methods*: Tumour samples were taken at surgery and cultured using the explant technique. Cells were reseeded and incubated with various concentrations of chemotherapy drugs. The MTS cytotoxicity assay was carried out to ascertain the response to the drugs and correlated retrospectively to the clinical outcome. *Results*: Tumours of similar stage and grade displayed heterogeneity in their responses to the same drugs. One hundred and thirty nine out of 144 tumours yielded chemosensitivity data, giving an evaluability rate of 96.5%. Of these, 76, mainly ovarian specimens, were available for *in vitro-in vivo* correlations. *in vitro* sensitivity was associated with clinical response in 29 out of 39 patients. *in vitro* resistance was associated with progressive disease or death in 36 out of 37 patients. The association between *in vitro* and *in vivo* response, as measured by Chi-squared was highly significant, $p<0.0001$. *In vitro* response remained an independent prognostic indicator in multivariate analysis using Cox regression for progression-free survival but not for overall survival. *Conclusion*: Based on the results of this retrospective study, a randomised prospective trial using the explant technique in combination with the MTS assay should be carried out to validate chemosensitivity / resistance testing.

COMPARATIVE ANALYSIS OF DNA STRAND BREAKAGE AND POINT MUTATION INDUCTION

Sinéad Devery and P.T. Tomkins

Centre for Biopolymer and Biomolecular Research, Athlone Institute of Technology, Athlone, Co. Westmeath, Ireland. E-mail: SDEVERY@ait.ie.

Genotoxicity evaluation is a critical element of pre-clinical product assessment (1). DNA is the recipient of both macrolesion single -and double- strand breaks and microlesion single nucleotide mutations. Since the completion of the human genome project (HGP), special emphasis is being placed on the identification of single nucleotide polymorphisms (SNPs) due in part, to their association with disease susceptibility and pharmacological phenotypes. As part of a larger programme to develop rapid, organotypic, mechanistic screening assays for genotoxicity evaluation based upon mutation and strand breakage data, we have optimised the single cell gel electrophoresis (SCGE) or Comet assay for the detection of clastogenic DNA damage and repair in primary hepatocytes, keratinocytes and comparable continuous cells. We have also employed the comet assay to investigate the influence of the extent of strand methylation on hydrogen peroxide (H_2O_2) genotoxicity. The Comet assay detects DNA strand breakage by imaging fluorescently-stained DNA fragments mobilised in an electric field (2). Mutations in HOX2B and p53, following whole cell exposure to the same clastogen, have been detected in PCR products, using the glycosylase-dependent, BESS T-Scan™ assay. In the SCGE assay, cells embedded in agarose on GelBond film were subject to lysis, alkaline unwinding and electrophoresis at 0.8 V cm^{-1} and 350 mA for 5 min at pH >12.6. Resultant DNA comets were stained with ethidium bromide and tail moment resolved by image analysis (Perceptive Instruments). For BESS T-Scan™ the incorporation of dUTP during PCR amplification and subsequent treatment with uracil-N-glycosylase generated a series of nested fragments similar to a T-lane sequencing ladder when resolved on a standard sequencing gel. Both primary and immortalised cells showed similar basal control levels of strand breakage. For liver there is good agreement between primary (MR_{50} 353 μM) and continuous cells (MR_{50} 306 μM) for the incidence of DNA strand breakage following oxidant exposure. Conversely for skin primary mouse keratinocytes were 6X more resistant to H_2O_2-induced clastogenesis than XB-2 cells (MR_{50} of 181 μM and 30 μM, respectively). Hepatocytes were also more repair proficient, with tail moment values returning to levels similar to untreated controls, following 24-h post-treatment incubation at 37˚C. MR_{50}'s expressed as the H_2O_2 concentration, which caused a 50% elevation in comet tail moment. PCR-based mutation screening of a 1.1 kb region of the human HOX2B gene and p53 exons 7 and 8 revealed similar tissue and dose-dependent trends, obviously proportional to the restricted genome target size (3-4). It appears sensitivity to H_2O_2 is partially dependent on the degree of transcriptional exposure and we now report a means by which clastogenesis and unrepaired base damage can be detected simultaneously.

References

1 Devery S and Tomkins PT: Irish Scientist *6*: 69, 1998.
2 Klaude M *et al*.: Mutat Res *363*: 89-96, 1996.
3 Devery S and Tomkins PT :Toxicol Lett *95*: 38, 1998.
4 Devery S and Tomkins PT: Mutagenesis *14(6)*:641, 1999.

THE ROLE OF TAURINE IN CD3/IL-2-STIMULATED JURKAT ACTIVATION-INDUCED CELL DEATH

Stephen Maher, Claire Condron, David Bouchier-Hayes and Deirdre Toomey

Dept. of Surgery, Royal College of Surgeons in Ireland, Beaumont Hospital, Dublin 9, Ireland. E-mail: smaher@rcsi.ie

Introduction: Interleukin-2 (IL-2) is used in immunotherapy of melanoma and renal cell carcinoma and results in a

transient reduction in circulating T-lymphocytes. The mechanism underlying this reduction in lymphocytes is unknown. *in vitro* exposure to anti-CD3 mAb and IL-2 results in T-cell activation-induced cell death, which is mediated *via* apoptotic cytokines such as FasL, TNFα and TRAIL. Intracellular taurine levels are known to be important in regulation of T-cell death. We hypothesise that CD3/IL-2-stimulation increases expression of death-inducing cytokines, which results in T-cell death, and that taurine can attenuate cell death through modulation of expression of these cytokines. *Methods*: Jurkat T-cells (some preloaded with taurine) were stimulated with anti-CD3 mAb and IL-2. Fas, FasL, IL-2R, TNF-RI, TNF-RII expression and apoptosis were assessed *via* flow cytometry. TNFα production was measured by ELISA. Expression of TRAIL, TRAIL-RI and TRAIL-R2 was measured by Western immunoblotting. *Results*: Stimulation of Jurkat cells with CD3/IL-2 increased expression of FasL, TNF-RI, TNF-RII expression and induced apoptosis. Stimulation also resulted in cellular release of cytotoxic TRAIL, but did not result in production of TNFα. Pre-loading cells with taurine significantly reduced FasL expression and apoptosis. Inhibition of FasL resulted in a similar attenuation of apoptosis compared to that observed with taurine. TNF receptor blocking *via* neutralising antibodies did not alter apoptosis induced *via* CD3/IL-2 stimulation. *Conclusion*: Cellular stimulation with anti-CD3 mAb and IL-2 induces apoptosis. This cell death is mediated *via* the FasL and TRAIL apoptotic pathways, but not the TNFα pathway. Pre-loading Jurkat cells with the amino acid taurine attenuates apoptosis *via* a FasL-dependent mechanism. Taurine may be of use in reversing the lymphopenia observed with IL-2 therapy. FasL and TRAIL may be therapeutic targets in diseases, such as cancer, where the manipulation of AICD is desirable.

PYRROLO-1,5-N-BENZOXAZEPINES INDUCE APOPTOSIS IN CHRONIC MYELOID LEUKEMIA AND B-CHRONIC LYMPHOCYTIC LEUKEMIA CELLS

A.M. McElligott[1], M.M. McGee[2], G. Campiani[3], A. Ramunno[4], V. Nacci[3], D.C. Williams[2], D.M. Zisterer[2], A. Hayat[1], P.V. Browne[1] and M. Lawler[1]

[1]Department of Haematology, Sir Patrick Dun Research Laboratory, Central Pathology Laboratory, St James's Hospital, Dublin 8; [2]Department of Biochemistry, Trinity College, Dublin 2, Ireland; [3]Dipartimento Farmaco Chimico Tecnologico, Universita' Degli Studi di Siena, Siena; [4]Dipartimento di Scienza Farmaceutiche, Universita' Degli Studi di Salemo, Fisciano, Italy. E-mail: mcelliga@tcd.ie

Chemotherapeutic agents are used widely in the treatment of haematological malignancy. However certain leukemias are resistant to chemotherapy-induced apoptosis, therefore the development of novel apoptosis-inducing agents is required. Previously we have shown that a series of novel pyrrolo-1,5-benzoxazepines potently induce apoptosis, as shown by cell shrinkage, chromatin condensation, DNA fragmentation and PARP cleavage, in three CML cell lines, K562, KYO.1 and LAMA 84. This apoptotic response involves the early activation of the stress-activated protein kinase, c-Jun N-terminal kinase (1). B-chronic lymphocytic leukemia (B-CLL) is one of the most common types of leukemia in the western world and is primarily caused by defects in the apoptotic mechanism.

The aims of this study were to examine the effects of PBOX compounds on cells cultured from B-CLL patients and haematopoietic cells from normal donors taken at bone marrow harvest.

Peripheral blood cells from B-CLL patients and bone marrow cells from normal donors were cultured with PBOX compounds and tested for viability and apoptosis induction.

PBOX compounds induced apoptosis in B-CLL cells in a dose-dependent manner, with IC_{50} values of less than 1μM. PBOX compounds did not induce apoptosis or affect the proliferative capacity of normal donor bone marrow progenitor cells as determined by colony forming unit granulocyte macrophage assays (CFU-GMs).

These results indicate that further investigation of the use of PBOX compounds in the induction of apoptosis in chemotherapy-resistant haematological malignancies such as CML and CLL is warranted.

References

1 McGee MM, Campiani G, Ramunno A, Nacci V, Lawler M, Williams DC and Zisterer DM: J Biol Chem *277*: 18383-18389, 2002.

CYTOCHROME P450 EXPRESSION AND SENSITIVITY TO ANTICANCER AGENTS

Vanesa Martinez, Robert O'Connor and Martin Clynes

National Institute of Cellular Biotechnology, Dublin City University, Dublin 9, Ireland. E-mail: vanesa.martinez2@ mail.dcu.ie

Development of drug resistance has become a major limitation for chemotherapeutic treatment of cancer. One of the mechanisms displayed by resistant cells is to reduce drug activation or enhance its detoxification. Cytochromes P450 are able to metabolise a number of chemotherapeutic drugs into less or non-toxic derivatives. We have evaluated the possibility of these enzymes influencing the sensitivity of tumour cells to anticancer agents. For this we have chosen two cell lines, the

Caco-2 cells, derived from a human colonic adenocarcinoma cell line, and the A549 cells, obtained from a human lung adenocarcinoma. Both cell types express different P450 isoforms and they are metabolically competent. The purpose of the present work is to study the changes in the P450 expression profile after exposure to chemotherapeutic drugs, and also the relationship between these alterations and cell sensitivity to these drugs.

MODULATION OF DIFFERENTIATION IN POORLY-DIFFERENTIATED LUNG CARCINOMA CELLS FOLLOWING EXPOSURE TO THE THYMIDYLATE SYNTHASE INHIBITOR 5-FLUOROURACIL AND ITS ANALOGUES 5 FLUORO 5'-DEOXYURIDINE AND 5 FLUORO 2'-DEOXYURIDINE

William Bryan, Paula Meleady, Jason McMorrow and Martin Clynes

National Institute for Cellular Biotechnology, Dublin City University, Dublin. 9, Ireland. E-mail: william.bryan2@dcu.ie

The fluoropyrimidine, 5-fluorouracil (5-FU), is an anti-metabolite drug that is widely used for the treatment of cancer, particularly for colorectal cancer. It exerts its anticancer effects through inhibition of thymidylate synthase (TS) and incorporation of its metabolites into RNA. The pyrimidine analogue of 5-FU, a 5-FU prodrug -5 Flouro 5'-deoxyuridine, and a metabolic derivative of 5-FU (5 Flouro 2'-deoxyuridine), were assessed as potential differentiation modulators in the poorly-differentiated lung carcinoma cell lines, DLKP and A549. Previous studies have shown that treatment of these cells with the differentiation-modulating agent 5-bromo-2'-deoxyuridine (BrdU) can induce the expression (at protein level) of 8, 18 and 19, and β1-integrin at a post-transcriptional level. This post-transcriptional up-regulation by BrdU was found to coincide with increased expression and phosphorylation of the translation initiation factor, eIF4E. In this study, cells were exposed to the 3 fluorinated drugs for up to 14 days and increased levels of keratins 8, 18 and 19, as well as eIF4E, were observed in treated cells. Altered adhesive properties in the above cell lines to the extracellular matrix (ECM) proteins, laminin, collagen and fibronectin were also observed.

A COMPARATIVE STUDY OF EFFECTS OF CONJUGATED LINOLEIC ACID ISOMERS ON PROLIFERATION IN MCF-7 AND DRUG-RESISTANT MCF-7 BREAST CANCER CELLS

Isobel O'Reilly and Rosaleen Devery

National Institute for Cellular Biotechnology, Dublin City University, Dublin 9, Ireland. E-mail: rosaleen.devery@dcu.ie

Several conjugated isomers of linoleic acid both in pure form and as a mixture have been shown to be cytotoxic at micromolar concentrations for a variety of breast, colon, prostate and skin tumour cell lines (1). Cytotoxicity of conjugated linoleic acid (CLA) was found to be concentration -dependent in a human breast cancer cell line (MCF-7) and was associated with peroxidative damage (2), rapid cellular uptake and modulation of membrane phospholipid composition. Exogenous unsaturated fatty acids that modulate membrane composition may also modulate tumour cell sensitivity to chemotherapeutic agents. *Aims*: The purpose of this work was to compare the sensitivities of an MCF-7 parental cell line and an MCF-7 cell line resistant to cisplatin to three preparations of CLA: a mixture of isomers containing 29.5% *c*9*t*11 and 29% *t*10*c*12-CLA and two separate isomers, *c*9,*t*11-CLA and *t*10,*c*12-CLA. *Methods*: Stock solutions of sodium salts of fatty acids (71mM) were prepared in medium containing bovine albumin (0.16mM). MCF-7 and MCF-7cisplatin-resistant cell lines were grown in Dulbecco's Minimum Essential Medium supplemented with foetal bovine serum (5 % by vol), 1 mM HEPES, 10 mM pyruvate and 1 unit/ml penicillin/streptomycin. Cells were plated into 96-well microtitre plates at a density of $5x10^3$ cells/well (for 1 and 3 days) and $1x10^3$ cells/well (for 5 and 7 days). Cells were cultured for 24h before adding 100 μl of medium containing fatty acids in 7.1 μM increments to a final concentration of 71 μM. Cytotoxicity was assessed after 1,3, 5 and 7 days using the One Solution Cell Proliferation Assay (Promega). Data was obtained from at least three experiments in which treatments and time points were set up in triplicate. *Results*: Cytotoxic effects of conjugated linoleic acid mixture, *c*9,*t*11-CLA and *t*10,*c*12-CLA were time- and dose-dependent in both cell lines. Significant inhibition ($p<0.05$) of cell growth was observed only after 5 and 7 days of treatments. The IC_{50} in MCF-7 cisplatin-resistant cells after 7 days of treatment with CLA mixture of isomers, *c*9,*t*11-CLA and *t*10,*c*12-CLA was 21.4 μM, 42.8 μM and 17.8 μM, respectively. Corresponding IC_{50} values in MCF-7 cells were 36.4μM, 42.7μM and 34.9μM. By contrast, negligible effects on growth were observed following exposure of both cell lines to linoleic acid at concentrations <64.2 μM. Linoleic acid at 71 μM inhibited growth by 41% (n=4) ($p<0.05$) following 7-day exposure of MCF-7 cisplatin-resistant cells and by 25% (n=3) ($p=0.054$) following 5-day exposure of MCF-7 cells. *Conclusion*: Cytotoxicity of CLA is dependent on isomer composition, concentration and duration of exposure. MCF-7 cisplatin-resistant cells were more sensitive to inhibition by *t*10,*c*12-CLA and the CLA mixture than parental MCF-7 cells. Data suggest that CLA isomers are likely candidates for investigating modulation of tumour cell chemosensitivity.

References
1 Belury *et al*: Ann Rev Nutr *22*: 505-531, 2002.
2 O'Shea *et al*: Anticancer Res *20*: 3591-3602, 2000.

A COMPARISON OF THE UPTAKE AND EFFLUX OF SELECTED ANTHRACYCLINE CYTOTOXIC DRUGS IN A LUNG CARCINOMA CELL LINE

Michael O'Leary[1], Robert O'Connor[2], John Crown[1] and Martin Clynes[2]

[1]Dept. of Medical Oncology, St Vincent's University Hospital, Elm Park, Dublin 4; [2]National Institute for Cellular Biotechnology, Dublin City University, Dublin 9, Ireland.
E-mail: maoleary@hotmail.com

Many years after the earliest recognition of the existence of such a phenomenon, the cytotoxic therapy of malignant tumours remains seriously hampered by multi-drug resistance. The screening of compounds in the development of drugs to overcome this problem requires the use of preclinical models of human cancers. The simplest (and cheapest) models are tissue culture based. 'Groups' or 'Families' of drugs can be tested using cell culture techniques. We know, however, from clinical experience that agents of the same family, while very similar structurally, behave very differently clinically with relation to their pharmacokinetics, toxicity and clinical efficacy. We believe it would be informative to compare the handling of several closely related drugs from the same family by a single cell line. We performed experiments to examine and compare the uptake and efflux of three anthracycline antibiotics (doxorubicin, daunorubicin and epirubicin) in a lung cancer cell line, DLKP. The DLKP cell line is derived from an undifferentiated human non-small cell (squamous) lung cancer. Our findings suggest that data resulting from work with a single drug cannot be assumed to hold for other members of that drug family, no matter how close the structural relationship. We plan to expand the experiment to examine the handling of these cytotoxic agents by other cell lines and to repeat these experiments with other closely related cytotoxic drugs.

ADRIAMYCIN ALTERS THE EXPRESSION OF DRUG EFFLUX PUMPS AND CONFERS AMPHOTERICIN B TOLERANCE IN *CANDIDA ALBICANS*

Joseph O'Keeffe, Sean Doyle and Kevin Kavanagh

Medical Mycology Unit, National Institute of Cellular Biotechnology, Department of Biology, National University of Ireland Maynooth, Co. Kildare, Ireland

Cancer is a multi-factorial disease that attacks indiscriminately across all demographic areas of society. Coupled with the debilitating nature of the condition is severe immuno-suppression which allows infection by a range of viral, bacterial and fungal pathogens. The yeast *Candida albicans* is capable of causing a range of superficial and systemic diseases in susceptible hosts and, in cases of severe immuno-suppression, can prove fatal. Conventional treatment of systemic fungal infections relies upon the use of amphotericin B along with a variety of other anti-fungal agents.

The successful treatment of cancer with anti-neoplastic agents such as adriamycin may be hampered by inefficient drug delivery to the target site or by circumvention of the action of the anti-neoplastic agent. Patients may manifest resistance to a range of anti-neoplastic agents due to inherently resistant tumours or the inadvertent selection for resistance in initially susceptible tissue. Common resistance phenotypes may be due to alteration in drug target sites and/or enhanced cellular efflux. Increased expression of a number of drug efflux pumps including MDR1 and MDR3 (multi-drug resistance pump) have been implicated in conferring resistance in humans.

The aim of the work presented here was to establish whether exposure of the yeast *C. albicans* to adrimaycin altered the expression of drug efflux pumps and consequently altered the susceptibility of the yeast to amphotericin B. Using monoclonal antibodies directed against human MDR 1 and 3, and polyclonal antibodies against CDR 1 (*Candida* drug resistance) we demonstrate that adriamycin induces an elevation in the expression of these efflux pumps which, together with previously recorded alterations in the composition of the fungal cell membrane, may confer tolerance to amphotericin B.

This work highlights the fact that adriamycin therapy may inadvertently alter the susceptibility of *C. albicans* to amphotericin B which may have deleterious consequences for anti-cancer chemotherapy regimes incorporating this anti-neoplastic agent.

Acknowledgements
This work is supported by funding from the HEA under PRTLI 3. JOK is the recipient of an Enterprise Ireland Postgraduate Student Bursary.