oral presentations

6.6 Evaluation of Drug Combinations as a Strategy to Eradicate Quiescent Leukaemic Stem Cells in Chronic Myeloid Leukaemia

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The development of a small molecule antagonist of BCRABL, the fusion oncoprotein that is thought to be causative in Chronic Myeloid Leukaemia (CML), heralds a new era in the treatment of this otherwise refractory disease. However, possible mechanisms of resistance to Glivec® (imatinib mesylate, Novartis, Switzerland) have been described including gene amplification and P-glycoprotein up-regulation. We have previously demonstrated the existence of quiescent (G0), leukaemic (Philadelphia, Ph+), stem (G0) and normal (null) CML samples studied. Moreover, these cells have been shown to be insensitive to Glivec® in vitro, even at a concentration 10-fold higher than normally achieved in patient plasma (Graham et al., Blood 2002; 99(1): 335). Indeed, in the presence of growth factors, the percentage recovery of input CD34+ cells in the G0 fraction was significantly greater in the presence of Glivec® than in the absence of drug, suggesting an accumulation of G0 cells, an effect modulated by the drug itself. Further, Ph+ CD34+ G0 cells have been identified by FACS in a minority of patients as undivided and divided. Interestingly, both Ara-C and FTI individually caused an apparent greater accumulation of G0 cells than Glivec® alone. Moreover, Ara-C in combination with Glivec® elicited a further 38% increase in number of viable, leukaemic stem cells than with Ara-C alone. Further experimental compounds will be tested in this system, namely LY294002 (CN Biosciences), PEG-Intron (Schering) 17 AAG and Byrostatin (both NCI, Bethesda, MD).

6.7 Comparisons of Prognostic Factors in Patients Participating in Phase I Clinical Trials with Conventional Cytotoxic Drugs versus New Non-cytotoxic Agents

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Aims: Little is known about prognosis of patients entering phase I trials. The objectives of this retrospective study were: (1) to identify prognostic variables for toxicity and survival in patients (pts) participating in phase I clinical trials a single centre; (2) to compare characteristics of pts treated with cytotoxic chemotherapy (CT) and non-cytotoxic drugs (non-CT); (3) to determine whether being treated by non-CT might have any impact on prognosis or toxicity.

Patients and methods: Data were collected from 420 (114 CT, 306 non-CT) pts enrolled in 16 phase I trials (5 CT and 11 non-CT trials) in our institution between 1991 and 2000. Pt characteristics: 210M, 210F, median age 56 yrs (22-87), median PS 1 (0-3), median duration of treatment 57 days (2-393). The chi-square test and Mann-Whitney test were used to compare treatment groups. Univariable analyses of survival times were performed using the log-rank test. Cox proportional hazards model was adopted to estimate prognostic factors in overall survival (OS). The Prognostic Index (PI) was generated from data on all 420 pts. A logistic regression model was used to explore predictive variables of toxicity. All studies were approved by the Central Oxford Research Ethics Committee.

Results: Overall tumour response: 4.5% (95% CI: 2.7-7.0%); Median OS: 202 days (95% CI: 189-249). Multivariate analysis showed that pts with better PS, high Hb, WBC or LDH in normal range and fewer sites of metastases had significantly better OS. Males, pts with low platelet count, high WBC count or treatment with a non-CT phase I agent had significantly less chance of toxicity. Comparing CT with non-CT treated groups: the CT group had a higher proportion of females (p < 0.001), were younger (p = 0.04), had better PS (P=0.02), were more likely to have liver involvement (p<0.001) and have LDH in normal range (p<0.001). Pts in the CT group had a longer duration of treatment (median time on study 73 vs 51 days, p<0.001), better tumour response (1 CR and 15 PR versus 3 PR, p<0.001). Median survival was not significantly different between the CT and non-CT groups (260 vs 192 days, p = 0.47). In pts with liver metastases (n = 127) median survival was significantly shorter in the non-CT group (137 vs 228 days, p = 0.02).

Conclusion: Multivariate analysis supports the view that high LDH or WBC, low Hb, poor PS and a higher number of metastases are independent adverse prognostic variables for survival. Female, treatment with phase I CT drugs, low baseline WBC and high platelet counts are independent predictors of toxicity. Cytotoxic agents, compared to non-CT, are more likely to induce a toxic response, although OS is not affected by the class of agent. We conclude that entry into a phase I trial of a non-CT drug is a safe option in this population of usually heavily pre-treated pts. The PI generated from these data could be used to estimate the survival probability for pts entering phase I studies in the future.

6.8 Aim High – Adjuvant Interferon in Melanoma (High Risk): The Costs.

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Analysis of tumour tissue demonstrated that selective virus replication had occurred. In the trial reported here, patients with high grade glioma had their tumours surgically resected and 10^7 pfu of the HSV1716 injected into tissue adjacent to the cavity. This allowed assessment of safety HSV1716 injection into normal brain. Of the twelve patients recruited, seven had recurrent disease, and five were newly diagnosed. Eleven patients received further treatment, either radiotherapy or chemotherapy. No evidence of toxicity associated with HSV1716 has been detected one patient who became ill with pyrexia of unknown origin (six weeks following HSV1716) but with no evidence of systemic herpes simplex infection, clinically or immunologically was given intravenous Acelvolir as a precautionary measure.

Two of the patients with recurrent glioblastoma have died due to disease progression at two and six months post virus injection, and four of the remaining ten patients have shown clinical and radiological evidence of tumour regrowth. One of the newly diagnosed patients who showed evidence of tumour regrowth has undergone further surgical resection. The remaining six patients are presently well at 7 weeks to 9 months post virus administration. This study has shown HSV1716 into tumour cavity to be a safe approach. Subsequent time to progression data will provide evidence of potential efficacy. The information obtained from this and previous studies allows informed design of planned Phase 2 efficacy trials.
The primary objective of this randomised trial was to compare the effect of treatment with Interferon alpha-2a with no further therapy on overall survival (OS) and event free survival (EFS) of patients with high risk (stage IIB, III) melanoma. Secondary objectives were to study the interaction of Interferon with age and gender, to document the side effects of long term administration of Interferon and to assess economic implications. Between 3rd October 1995 and 22nd December 2000, a total of 674 patients were recruited from 37 centres in the UK. Recent survival analysis confirms the preliminary findings. As of 31.01.02, overall survival was 43% and 41% and relapse free survival 34.5% and 30% for Interferon and control respectively.

Methods - The economic sub-study collected data from a one-in-five sample of trial patients across all recruiting centres. A total of eighty-two patients were entered, 40 in the control group and 42 in the intervention group. Use of health services resources were collected from patients using a self-completed questionnaire. Utility estimates were collected using the EQ-5D (EuroQol) combined with survival data to estimate quality adjusted life years (QALY).

Results - In the first few months of the trial total costs are considerably higher in the interferon group, due to the cost of interferon (at 6 months mean costs in the control group were £1,491, compared to £3,255 in the interferon group). However, this difference reduces slightly over time due to reduced hospital costs, such that mean costs at 24 months in the control and interferon groups are £6,367 vs. £7,865, respectively. This pattern appears to be followed by the quality of life results produced by the EQ-5D. QALY differences are small, but in favour of the control group over the first 12 months (81.8 vs. 79.7 QALYs), whilst at 24 months the interferon group has higher quality of life, although this is based on small patient numbers (<20).

Conclusion - Despite the high costs of therapy, these preliminary results point to some cost offsets associated with treatment. Also, any adverse impact on health-related quality of life during treatment appears small. However, as yet there appears to be no survival benefit with interferon.

7.2 GLUT-1 AND CAIX AS INTRINSIC MARKERS OF HYPOXIA IN CANCER: RELATIONSHIP TO PIMONIDAZOLE BINDING

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The presence of hypoxia in tumours results in the overexpression of certain genes, which are controlled via the hypoxia-inducible factor (HIF-1). HIF-1. Hypoxic cells are known to be radioresistant and chemoresistant, and a reliable surrogate marker of hypoxia is desirable to ensure that treatment with radiotherapy or hypoxia-selective bioreductive drugs may be rationally applied. Recently, the HIF-1-regulated proteins Glut-1 and CAIX were validated as intrinsic markers of hypoxia by comparison with pO2 measured using the Eppendorf oxygen electrode, which is believed to account for both acute and chronic hypoxia. Following on from this work, Glut-1 and CAIX have now been compared with the use of the bioreductive hypoxia marker pimonidazole. Pimonidazole (dose 0.5 g/m2) was administered to 42 patients with advanced carcinoma of the cervix, 16 hours before biopsy. Sections of single or multiple biopsies were then immunostained for Glut-1 and CAIX, and the area of staining scored by means of a low-tech "field-by-field" semi-quantitative averaging system. Using one biopsy only, Glut-1 expression correlated strongly with pimonidazole binding (r = 0.538, P < 0.001), whereas CAIX expression showed only a borderline correlation (r = 0.271, P = 0.083). Glut-1 and CAIX expression also correlated significantly (r = 0.399, P = 0.009). However, when multiple biopsies of each tumour were scored, Glut-1 expression showed considerably more heterogeneity between biopsies than CAIX. The pharmacokinetic properties of pimonidazole, together with the time taken between administration of pimonidazole and biopsy, means that pimonidazole binding in tumours, and consequently, any closely correlating marker, are more likely to provide a measure of chronic hypoxia. Thus, this study has shown that HIF-1 regulated genes, particularly Glut-1, have potential for future use as predictors of the malignant changes mediated by chronic hypoxia, and warrant further investigation as a indicators of response to bioreductive chemotherapy, surgery and hypoxia-mediated gene therapy.

7.3 HYPOXIA IN BLADDER CANCER: VALIDATION OF INTRINSIC MARKERS WITH PIMONIDAZOLE AND THEIR INFLUENCE ON CLINICAL OUTCOME AFTER ACCELERATED RADIOTHERAPY WITH CARBOGEN AND NICOTINAMIDE

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Tumour hypoxia limits the effectiveness of treatment for cancer. Encouraging results from the recent pilot study of accelerated radiotherapy with carbogen and nicotinamide (ARCON) in bladder cancer suggest hypoxia may be an important factor. The emergence of pimonidazole as a hypoxic marker has allowed quantification of hypoxia by immunohistochemical means in tumours accessible to biopsy. The putative intrinsic markers of hypoxia, Carbonyl Anhydride-9 (CA9) and Glucose transporter-1 (GLUT 1), have been validated.
Approval from the local ethics committee was obtained. Thirty-one patients with confirmed transitional cell carcinoma of the bladder received pimonidazole prior to transurethral resection of the tumour. Sections available from 26 tumours were double stained for pimonidazole (hypoxia) with Ki67 (proliferation), & pimonidazole with CD31/34 (vasculature). Sections were also stained for GLUT 1 and CA9. Sections were analysed with the aid of image analysis software. 

The median hypoxic fraction assessed by pimonidazole was 9% (range 0 to 38%). The geographical match and fraction of tumour stained by GLUT 1 and CA9 was very similar to pimonidazole (correlation coefficient 0.86 and 0.76 respectively). Most staining was distant from vessels (median 99µm).

Some hypoxia was close to vessels (<40µm), suggesting a perfusion limiting mechanism to oxygen delivery in these areas. Dual staining for pimonidazole and proliferation showed a predominantly inverse relationship between these factors. However, some hypoxic regions of less than 40µm from a vessel had a higher proliferative index than those further away from vessels. 

Having confirmed GLUT 1 and CA9 as appropriate intramarker for hypoxia, archived paraffin embedded samples of bladder carcinoma from 64 patients treated with ARCON were obtained. Sections were stained for GLUT1, CA9, Ki67 and CD31/34 and analysed as above.

The median age of the group was 72.9 years (range 38 to 88 years). Six patients had T1 disease, 19 had T2 disease, 38 had T3 disease and one had T4 disease. Forty-five tumours were grade 3, 17 were grade 2 and 2 were grade 1. The median follow up time was 27 months.

The median stained fraction for GLUT1 was 6.5% (range 0 to 62%) and for CA9 was 3.5% (range 0 to 67%). Those patients above the median (more hypoxic tumours) for each marker had a statistically significantly worse cause specific survival (GLUT1 p<0.005, CA9 p<0.003) but not local recurrence or metastasis free survival, analysed with a log rank test. A multivariate analysis, accounting for stage, age, grade, Ki67 index and vascularity confirmed hypoxic fraction as an independent factor in cause specific survival (GLUT1 p<0.02, CA9 p<0.03).

In conclusion, hypoxia is a significant factor in bladder cancer, and can be quantified with both extrinsic and intrinsic markers. Staining patterns are consistent with diffusion limited and perfusion limited mechanisms of hypoxia. Proliferation is reduced in hypoxic areas, the greatest effect seen with increasing distance from vessels. In a cohort of patients with bladder cancer treated with ARCON, hypoxic tumours were associated with a significant reduction in cause specific survival.

7.4 A NOVEL PROMOTER-ENHANCER FOR HYPOXIA-SELECTIVE GENE THERAPY.

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We are developing a cancer gene therapy targeted to hypoxia, a unique feature of solid tumours, which is transcriptionally silent in normal tissues. The efficacy of transcriptional targeting with any specific promoter is directly related to the toxicity of the transgene being used. The development of tightly regulated specific enhancer/promoter elements is pivotal to deliver potent therapeutic genes to provide a clinically relevant treatment modality.

Hypoxic response is dependent upon the binding of the transcription factor HRE, a cognate receptor, the hypoxia response element (HRE). Using HREs derived from the mouse phosphoglycerate kinase (PGK) and lactate dehydrogenase (LDH) genes in combination with minimal promoter elements from the tumour associated carbonic anhydrase-9 (CA-9) we have developed a number of novel promoter enhancer combinations tightly controlled by hypoxia.

CA-9 is strongly inducible by hypoxia in a broad range of tumour cells, yielding levels of expression comparable to those achieved with full length SV40. This response is regulated by a HRE within the minimal promoter. The HRE-CA-9 elements were introduced into the pGL3-basic luciferase reporter vector and transiently transfected into a panel of 9 human tumour cell lines. The response of the vectors to air or anoxia (0.002% O2) for 16 and 40 hours was tested. A range of optimal HRE driven SV40 constructs (pGL3-Prom), and CA-9 alone vectors were tested in parallel for comparison. 

We have consistently seen a 6-fold reduction in spurious background expression following evaluation of the HRE-CA-9 constructs against their HRE-SV40 pairs. We have further enhanced anoxic induction by exploiting the unique positioning of the CA-9 HRE, 4bp from the transcriptional start site, to construct novel hypoxia regulated enhancer and promoter ‘fusions’. The replacement of the native CA-9 HRE with an optimal HRE composed of a triplicate LDH HRE (LDH3) resulted in up to a 30-fold enhancement of expression compared to the native CA-9. 

Whilst maintaining transcriptional silence in air we have achieved levels of induction comparable to our optimized vector, LDH3-SV40.

This application of the minimal CA-9 promoter provides both a further degree of hypoxia regulated, tumour-specific expression and an attractive alternative to viral promoters that can be shut down in vivo. This data demonstrates the ability of this novel vector to deliver a suitably high level of gene expression for gene therapy.

5.7 HYPOXIA SELECTIVE GENE DIRECTED ENZYME PRODRUG THERAPY.

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The aim of our research is to combine hypoxia driven gene therapy and bioreducive chemotherapy to selectively target therapeutically refractive hypoxic tumours. To this end we have developed a novel synthetic series of indolequinone pro-drugs similar in structure to the clinical bioreducive drug mitomycin c (mmc). upon evaluation of the bioreducive cytotoxicity of this series in aerobic and hypoxic conditions a lead compound was identified 5-aziridinyl-1-hydroxymethyl-1-methylindole-4,7-dione (629). we have compared the potency of 629 and its selectivity for activation by the reducing enzymes cytochrome P450 reductase (P450R) and dt-diaphorase (DTD) using stable cell lines, derived from a panel of human breast carcinoma cell lines, which over-express these enzymes.

| Cell line | Clone | Enzyme induction | IC50 (µM) | HCR |
|-----------|-------|-----------------|----------|-----|
|           |       |                 | Air      | Anoxia |
| MDA468    | WT    | -               | 34.82    | 0.268 130  |
| p450r     | 8     | 2.526           | 0.00408  | 619   |
| DTD       | 115   | 0.02            | 0.00446  | 4.9   |
| T47D      | WT    | -               | 11.2     | 0.57   19.6  |
| p450r     | 60    | 0.49            | 0.0093   | 52.7   |
| DTD       | 278   | 0.18            | 0.025    | 7.3    |
| MDA231    | WT    | -               | 25.6     | 2.56   10  |
| p450r     | 28    | 2.15            | 0.036    | 59.7   |
| DTD       | 935   | 0.0225          | 0.0133   | 1.7    |

The data above suggests that 629 is preferentially activated through one electron reduction under conditions of low oxygen making it an ideal prodrug for an hypoxia driven GDEPT approach. To activate this novel pro-drug we have constructed adenoviral vectors encoding for constitutive P450R expression (Ad CMV P450R) and two vectors encoding for hypoxia induced P450R expression. These vectors ad PGK P450R and Ad LDH P450R contained a CA9 responsive promoters derived from phosphoglycerate kinase and lactate dehydrogenase a, respectively. We have demonstrated that all three viruses infect the breast tumour cell lines with high efficiency (40-80% transduction with moi 300). Using ad CMV P450R a 13 to 40 fold over-expression of P450R can be achieved compared to uninfected cell levels. We have also demonstrated a 5-10 fold increase in P450R levels specifically restricted to cells infected with Ad PGK P450R and Ad LDH P450R that have been exposed to hypoxia. These vectors are currently being evaluated.
for their ability to sensitise hypoxic tumours to both 629 and MMC in vitro and in vivo.

7.6 VIRUS-DIRECTED ENZYME PRODRUG THERAPY (VDEPT): CLINICAL TRIALS WITH ADENOVIRAL NITROIMIDAZOLE REDUCTASE (Ad-ntr)

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The bacterial enzyme nitroreductase (ntr) converts the prodrug, CB1954, to a highly toxic, short-lived, bifunctional alkylating agent. Since there is no human homologue of ntr, a VDEPT approach utilising ntr / CB1954 is feasible.

We have constructed a replication deficient adenovirus (Ad-ntr) that expresses ntr from the non-selective CMV promoter. Using Ad-ntr we have demonstrated in vitro activity, with a marked bystander effect, in a range of tumour cell lines. We have also demonstrated in vivo activity in subcutaneous xenograft models following intratumoural injection of Ad-ntr followed by systemic injection of CB1954.

A phase I trial to determine the maximum tolerated dose of Ad-ntr was undertaken in patients with hepatic metastatic colorectal cancer awaiting hepatic resection. Escalating doses of virus (1.05x108 – 1.05x1010) have been administered into the tumour percutaneously under ultrasound guidance. Three patients were treated at each dose level and additional patients were recruited if there was significant (>grade 2) toxicity. Liver resection was performed 48-96 hours after virus injection following which the tumour was resected. 11 patients have entered this study (virus particle dose of 108, n=3; 109, n=3; 1010, n=5). Toxicity has been minimal (1 patient had systemic flu-like symptoms) and there is a dose-related increase in PCR aided detection of viral DNA in venous blood. Neutralising antibodies to Ad-ntr are formed but there is significant inter-patient variation in kinetics and antibody class. Ntr is detected in resected tumours (1-3% of cells) with a dose-dependent increase in cell positivity. We have not yet reached our target percentage of cancer cell population staining positively for ntr, at which stage we will co-administer CB1954 at a dose ascertained in an earlier phase I and pharmacokinetic study (24mg/m2 IV).

Ad-ntr has also entered clinical trials of intratumoural injection in head and neck and prostate cancer.

7.7 DIRECT INJECTION OF ADENOVIRUS FOR ENZYME PRODRUG THERAPY OF PROSTATE CANCER

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Introduction: the ease of direct access to the prostate by transperineal, transurethral and transrectal routes makes locally targeted therapies for prostate cancer a viable option. We are undertaking a clinical trial of gene therapy administered by direct intraprostatic injection of adenovirus followed by intravenous injection of prodrug. The transgene e. Coli nitroreductase (ntr) has been inserted into a replication deficient adenoviral gene therapy vector, called ct1102, under the control of the cytomegalovirus immediate early promoter. Ntr converts the weak monofunctional alkylating agent 5-aziridinyl-2,4-dinitrobenzamide (ch1954) into a highly cytotoxic bifunctional alkylating agent that kills both replicating and non-replicating cells. In preclinical models, intratumoral ct1102 injection of subcutaneous pc-3 tumours in nude mice, followed by systemic ch1954 resulted in inhibition of tumour growth. The study involves: (1) a marker study to optimise a clinical protocol for in vivo prostate ntr expression, (2) investigation of the effects of direct injection of escalating doses of ct1102, with ch1954, into locally recurrent prostate cancer. Patients & methods: virus tropism for the prostate was verified by ex vivo injection of thin slices of freshly resected prostate tissue with a related virus expressing green fluorescent protein (ad-gfp) in place of ntr. The trial is divided into two parts; firstly the marker study involves a dose escalation phase i investigation in patients scheduled to undergo radical prostatectomy. Secondly, we will investigate a combination of virus and prodrug in patients with locally recurrent disease. The patients will undergo intraprostatic injection of ct1102 under transrectal ultrasound control in escalating doses, starting from 1010 adenoviral particles, rising to 1013 particles, in cohorts of three patients, with subsequent radical prostatectomy at 48-72 hours post injection. Ntr expression in the resected tissue is assessed by immunohistochemistry. The dose escalation is aimed at establishing the dose of ct1102 at which significant transgene expression is detected as well as conventional phase i toxicity endpoints. Results: in vitro culture of fresh prostate tissue with adenovirus expressing gfp revealed diffuse expression of transgene throughout glandular & stromal elements. 2 patients have now been treated with ct1102 in the marker study with 1010 particles of adenovirus with no serious adverse reactions. Trus images following intraprostatic injection demonstrated hypoechoic areas in the injected prostate lobe, indicating slow throughout the injected prostate lobe. Immunohistochemistry of prostate resected from the first patient has revealed transgene expression, apparently confined to glandular epithelium. Discussion: direct intraprostatic injection appears to be feasible and safe. Early results suggest that injected material disseminates via the prostatic ducts, the tissue of origin for prostate cancer. Evidence of virus infection and expression of the ntr protein has been seen in the lowest dose group. Dose escalation will proceed to administration of both virus and prodrug in locally recurrent cases.

7.8 RADIATION-ACTIVATED PRODRUGS: HYPOXIA-SELECTIVE RADIOLYTIC RELEASE OF POTENT CHLOROMETHYLBENZINDOLINE (CB1) CYTOTOXINS FROM NOVEL NITROARYLMETHYL QUATERNARY (NMQ) PRODRUGS

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Hypoxia is a major prognostic factor in radiation therapy. We have previously suggested the possibility of activating non-toxic prodrugs to potent cytotoxins by reducing them with ionizing radiation in the absence of oxygen. Reductive fragmentation of the nitroarylalkylated quaternary (NMQ) ammonium prodrugs of chloromethane (HN2) has been demonstrated by radiation under anoxic conditions, but the prodrugs are not sufficiently stable in vivo. The released chlorine mustard cytotoxin is not sufficiently potent for this mechanism to be therapeutically useful at the radiation doses used clinically. The aim of this study was to develop novel NMQ prodrugs capable of releasing significantly more potent chloromethylbenzindoline (CB1) cytotoxins on radiolytic reduction. Novel 5-amino- or 5-hydroxy-seco-CB1 cytotoxins were synthesized and demonstrated to have IC50 values (following 4 hr exposure) of ca.1 nM in a panel of human tumour cell lines. The corresponding 4-nitromidazolyl (4NI) and 2-nitropyrrrol (2NP) NMQ salts were 20-200-fold less potent, demonstrating significant deactivation in the prodrg form. When cells were exposed under anoxic conditions, IC50 values were 1.4 to 8-fold lower, indicating modest bioreductive (enzymatic) activation under anoxia. The hydroxy-NMQ-CB1 prodrugs had superior stability to the amino analogues in culture medium, as assessed by LC/MS, and were investigated further. When irradiated in anoxic formate buffer, these NMQ-CB1 prodrugs (20 µM) released their hydroxy-CB1 effects efficiently (HPLC analysis; G values 0.45-0.54 µmol J-1), indicating a stoichiometry close to one reducing equivalent. The 4NI and 2NP prodrugs (5 µM) were irradiated in anoxic human plasma and effector release was assessed by bioassay. These experiments demonstrated a 10 to 15-fold increase in cytotoxicity at 18 Gy, with an estimated G value for hydroxy-CB1 release of ca 0.02-0.08 µmol J-1. The extravascular diffusion properties of the CB1 and CB1 prodrugs in both anoxic and normoxic tumour model, while initial studies in mice indicate that the MTD values for the NMQ prodrugs are more than 10-fold higher than for the CB1 effector.
suggesting adequate metabolic stability in vivo. An investigation of their activity against hypoxic cells in HT29 tumors, in combination with radiation, is in progress.

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8.1 NOVEL DIRECT TARGETS FOR THE CONTROL OF GROWTH BY e-MYC

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The proto-oncogene product c-Myc lies at the interface between cell growth and division, impacting directly on both these processes. Indeed, Myc can stimulate cell growth (mass increase) independently of cell cycle effects. Since the bulk of a cell's dry mass is protein, growth is determined by the rate at which protein accumulates. Accordingly, Myc has been shown to stimulate the production of the protein synthetic apparatus. Activation of Myc causes increased synthesis of translation factors, ribosomal proteins and rRNA (1-3). We have found that c-Myc also activates the transcription of tRNA genes by RNA polymerase (pol) III. Thus, tRNA transcripts are rapidly induced by Myc in primary human fibroblasts. Furthermore, the expression of pol III products is significantly reduced following targeted disruption of the c-Myc gene. These effects reflect the direct action of c-Myc on the pol III machinery. Chromatin immunoprecipitation reveals the presence of c-Myc at tRNA genes in vivo. Moreover, c-Myc binds to the pol III-specific transcription factor TFIIIB. Inducing tRNA production is likely to contribute to the activation of protein synthesis that enables Myc to stimulate cell growth.

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8.2 DIFFERENTIAL EXPRESSION OF FIBROBLAST GROWTH FACTOR RECEPTOR SUBSTRATES IN HUMAN MALIGNANCIES

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Introduction

FGF receptor substrate (FRS), a membrane-anchored adapter protein targets signalling molecules to the plasma membrane in response to FGF stimulation. The two FRS isoforms, FRS2α and FRS2β (commonly referred to as FRS-2 and -3), are structurally similar and share 49% amino acid sequence identity. FRS is required for FGF-induced MAPK activation. We examined the levels of FRS-2 and -3 expression in a panel of human cancer cell lines and correlated their expression to FGF induced MAPK activation.

Materials and Methods

FRS-2 and -3 expression was detected by RT-PCR using specific primers. The following cell lines were included: prostate (LNCaP, PC3 and DU145), breast (MCF7), bladder (T24, J82 and RT112), melanoma(M2), colorectal (HT29) and osteoblastic (U2OS). FRS2 protein expression was further quantified following Western blotting. MCF7, LNCaP and J82 cells were selected for MAPK activation study, and the effect of FGF1 on ERK1/2 activation was quantified using phosphospecific antibody.

Results

FRS2 transcript was widely expressed and represents the predominant form of FRS expressed: 6/8 and 3/8 cell lines with detectable FRS-2 and FRS-3 expression, respectively. On Western blotting, LNCaP (prostate) and MCF7 (breast) cells expressed FRS2 at high level as a 90kD protein. T24 (bladder), M2 (melanoma), PC3 and DU145 (both prostate) expressed moderate levels of FRS2. FRS2 was expressed at low level in J82 (bladder) and HT29 (colorectal). The U2OS (osteoblastic) and RT112 (bladder) cells did not have any detectable levels of FRS2 expression. Using densitometry, corrected to α-tubulin expression, there was a 16 fold difference in FRS-2 expression between MCF7 and J82 cells. Consistent with the observed pattern of FRS2 expression, FGFI induced pERK1/2 level was highest in MCF7 cells, followed by LNCaP and was lowest in J82; their corresponding ratio of activation were 27, 13 and 7, when compared to serum starved controls. Of note, the three cell lines (MCF7, LNCaP and J82) did not have significant FRS3 expression.

Conclusion

FRS-2 appears to be the predominant form of FRS expressed in human malignancies. FRS-2, in the absence of FRS-3 expression, is adequate for FGF induced MAPK activation and the levels of FRS-2 expression correlates with the degree of FGF induced MAPK activation.

8.3 INVESTIGATING THE ROLE OF POLY(ADP-RIbose) POLYMERASE-1 IN THE RESPONSE OF GLIOMA CELLS TO LOW DOSES OF IONIZING RADIATION

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Introduction: The terms low-dose hyper-radiosensitivity (HRS) and increased radioreistance (IRR) describe the dual phenomena observed in many mammalian cell systems where small acute doses of radiation below ~50 cGy are more lethal per unit dose than higher doses up to ~2 Gy. There is evidence that HRS and IRR may reflect dose-dependent changes in DNA repair mechanisms. Recent work also suggests that the extent of HRS varies according to cell-cycle phase, being most marked in G2. Poly(ADP-ribose) polymerase-1 (PARP-1) is an abundant, highly conserved nuclear enzyme that rapidly binds to and is activated by DNA single and double strand breaks leading to recruitment and modulation of DNA repair processes. Evidence for a key role for this enzyme in the cellular response to radiation derives from PARP-1 knockout mice, and chemical and molecular inhibitors of PARP-1. Inhibition of PARP-1 was associated with sensitisation to radiation doses of 2 Gy and above (sensitiser enhancement ratio ~1.5) (ref). To date, however, the contribution of PARP-1 to the low-dose radiation response has not been extensively investigated. We are studying the effect of new, highly potent and specific inhibitors of PARP-1 on low-dose clonogenic survival of human glioma cell lines that display markedly differing degrees of HRS and IRR, with the dual aims of elucidating the role of PARP-1 in these phenomena, and investigating the potential for therapeutic symbiosis between PARP-1 inhibitors and an ultrafractionated radiation schedule.

Methods: Clonogenic survival of T98G (HRS+) and U373 (HRS-) human glioma cells following low-dose irradiation in the presence of the following chemical inhibitors of PARP-1 are being determined using a highly accurate fluorescence-activated cell sorting (FACS) clonogenic assay: 3-amino benzenamide, NU1025, 4-amino naphthalimide (4-ANI) and PJ34. All inhibitors have been tested at a range of concentrations to establish the optimum dose for modifying radiation response with minimal direct cytotoxicity. Further studies will investigate the effects of active drugs on the low-dose radiation responses of cells sorted according to cell-cycle phase.

Results: Preliminary studies utilising the first generation PARP-1 inhibitor 3-AB have demonstrated a modification of the low-dose radiation responses of both T98G and U373 cell lines that is complicated by the cytotoxic action of the chemical. NU1025 has exhibited an unexpected protective effect at low radiation doses (characterised by an enhancement in cell plating efficiency) that is being investigated.

Discussion: Exploiting the potency and specificity of new PARP-1 inhibitors will elucidate the role of PARP-1 in HRS/IRR, enabling assessment of the potential for a therapeutic interaction of such inhibitors with an ultrafractionated radiation schedule in the treatment of gliomas.
8.4
Nuclear BAG-1 expression predicts survival from breast cancer, and potentiates oestrogen dependent transcription.

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Background: BAG-1 is a multifunctional protein that binds a wide range of cellular targets including heat shock proteins and some nuclear hormone receptors. BAG-1 exists as three isoforms, BAG-1L, BAG-1M and BAG-1S. BAG-1L contains a nuclear localisation signal which is not present in the other forms and is predominantly located in the nucleus. 

Aims: To determine if BAG-1 expression is related to clinicopathological features in breast cancer, and to determine if BAG-1 modulates oestrogen dependent transcription.

Methods: Following ethical committee approval (Southampton & S. West Hants LREC Submission: 159/00), BAG-1 expression in tumours from 138 patients with breast cancer treated with hormonal therapy was assessed by immunohistochemistry. We performed multiplex quantitative real-time PCR assays to analyse the interaction of BAG-1 isoforms with oestrogen receptor (ER) alpha and beta, and reporter gene assays to measure the effects of BAG-1 isoforms on oestrogen dependent transcription.

Findings: Nuclear but not cytoplasmic BAG-1 immunostaining was associated with improved survival in patients treated with hormonal therapy (Hazard Ratios: BAG-1 nuclear positive: 0.334 (95% Confidence Interval 0.138-0.809), BAG-1 nuclear negative: 1; P=0.001). Nuclear BAG-1 expression was also moderately correlated with progesterone receptor (PgR) expression (r = 0.42) and ERα expression (r = 0.31). BAG-1L, but not BAG-1S or BAG-1M, interacted with both ERα and ERβ and increased oestrogen dependent transcription in ER positive MCF-7 breast cancer cells. BAG-1L overexpression stimulated transcription through both ERα and ERβ.

Interpretation: High levels of BAG-1L can increase responsiveness to oestrogens in breast cancer cells, and may be important in those cells for proliferation and survival. Similar to PgR and ERα status, BAG-1L may be a marker of responsiveness to hormonal therapy, via its direct effects on receptor function.

8.5
ASSESSMENT OF A PUTATIVE ONCOGENE ZNF217 WITHIN AMPLICON 20q BY MULTIPLEX QUANTITATIVE REAL-TIME PCR.

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Detection of gene amplification is a recognised process through which oncogenes can be identified. In this study we have coupled two powerful techniques, laser capture microdissection and multiplex quantitative real-time PCR to quickly and accurately assess the gene copy number of candidate oncogene ZNF217 in colon cancer. Several studies of colorectal cancer using comparative genomic hybridization (CGH) have identified chromosomal arm 20q, as the region most commonly gained in this tumour type. In addition high density FISH mapping analysis of the 20q amplicon in colorectal cell lines within our laboratory has identified ZNF217 to be contained within a region, 20q13.2 of high-level amplification. In this study we identified two cell nuclei containing some level of amplification.

To determine if ZNF217 amplification is a frequent event in colorectal cancer (48/81 tumours = 59.3%) and that the extent of its amplification varies markedly between tumours (range 3 – 13 copies). 1. Rooney PH, Murray GI, Stevenson DAI, Haines NE, Cassidy J, McLeod HL. 1999 Br J Cancer 80:862-873.

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8.6
GENE EXPRESSION PROFILE ANALYSIS OF TUMOUR-DERIVED CELL LINE ORIGINS EXPRESSING THE CANCER TUMOUR SUPPRESSOR GENE, ZNF217.

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Fibulin-1 is an extracellular matrix protein implicated as a suppressor of tumour cell adhesion, motility and growth. The mechanism by which fibulin-1 mediates these suppressive effects is unclear. Here, we used DNA microarray analysis to characterise a profile of genes whose expression is modulated by the two predominant fibulin-1 splice variants, fibulin-1C and D. The human breast tumour cell line MDA-MB-231 and the human lung fibrosarcoma cell line HT1080, both of which express little or no fibulin-1, were stably transfected to express fibulin-1C or D variants. Expression of the exogenous fibulin-1 variants was confirmed by immunoblot analysis of conditioned culture media. Using Affymetrix HuGeneFL arrays containing probe sets for 7,129 transcripts, gene expression profiles were determined in duplicate for both empty vector and fibulin-1 variant transfectants. Comparison of profiles obtained from empty vector- versus fibulin-1D-transfected MDA-MB-231 cells revealed 58 genes as being differentially expressed. Two hundred and thirty differentially expressed genes were identified in comparisons of empty vector- and fibulin-1D-transfected HT1080 cells. Furthermore, 28 genes were identified in comparisons of empty vector- and fibulin-1C-transfected HT1080 cells. Analysis of these profiles revealed evidence of fibulin-1-dependent effects on gene expression that included variant-specific effects. For example, expression of thrombomodulin-1 increased by ~2 fold in fibulin-1D-expressing cells (both MDA-MB-231 and HT1080), but decreased by 5.0 fold in fibulin-1C expressing HT1080 cells. In contrast, collagen alpha 1 type IV expression increased > 3.0 fold in cells expressing either fibulin-1 variant. OB-cadherin (cadherin 11), a calcium-dependent cell adhesion protein, was differentially expressed in fibulin-1 transfectants as compared to empty vector transfectants. Specifically, OB-cadherin expression was increased in the fibulin-1D-transfected MDA-MB-231 cells (+28.8 fold) and decreased in both the fibulin-1C (-9.4 fold) and D (-5.4 fold) transduced HT1080 cells. Transcripts for two other genes, ARPC4 (-3.8 fold) and RhoGDIBeta (+2.0 fold), were differentially expressed between the HT1080 fibulin-1C and empty vector transfectants. Based on these findings, it is hypothesised that fibulin-1 acts to (a) inhibit ARPC4 gene expression, with a consequent negative impact on cell motility, and (b) stimulate Rho GDIBeta gene expression, which in turn inactivates RhoGTPase-dependent pathways, resulting in decreased cellular motility and tumourigenic reversion. [Supported by the Health Research Board - Ireland, Enterprise Ireland and Cancer Research UK]
For certain carcinogens the pattern of DNA adducts produced in p53 has been shown to be related to the pattern of p53 mutation in associated human tumours; establishing causative links between the agents and cancers induced. Similarly, a positive correlation between patterns of p53 oxidative damage and mutation could help establish the cancer relatedness of oxidative DNA damage. Until recently it was not possible to compare induced damage with mutation in p53 in vivo, as no suitable mutation assay was available. However, in the present study we have compared the distribution of induced oxidative damage with the distribution of induced mutation in vitro in human p53 using LMPCR and a developed plasmid-based p53 forward mutation assay, respectively. Briefly, LMPCR permits the detection of rare DNA breaks along single copy genes at nucleotide resolution in the full human genome, and combining this technique with damage recognising E. coli Nth and Fpg proteins allows for mapping of oxidative base lesions. In the mutation assay, a plasmid bearing a full c-DNA copy of human p53 is damage-treated in vitro, then transformed into yeast cells containing an ADE2 gene under the control of a p53 protein-responsive promoter. Transformed colonies containing mutant p53 protein are unable to express the ADE2 gene and accumulate a red metabolite. The red colonies are picked, grown and the human p53 amplified and sequenced. Using LMPCR we have mapped Cu+H2O2-induced oxidative damage along the transcribed strand of exons 5 and 7 of p53 in isolated human DNA, and have compared this with the distribution of mutations induced by Fe+H2O2+ascorbate reactions in the plasmid c-DNA p53 exon 5 and 7 sequences. Even with the different gene targets and damaging agents used, there is a marked degree of similarity between the distribution of damage and mutation, particularly for exon 5 where the regions of greatest damage (exclusively manifest at guanines in codons 142, 149-156, 177-178) strongly correlate with the locations of highest mutation frequency (predominately GC/TA transversions and GC/AT transitions at codons 143, 151-154, 177). The similarity between the Cu-dependent damage and Fe-dependent mutation spectra is in line with metal ion dependent peroxide-induced damage being more sequence specific than metal specific. Future studies directly comparing the same gene-targets and damaging agents, will allow us to rigorously address which damaged sites are critical to mutation, and any correlation with the reported in vivo mutations in human tumours will greatly increase relevance of our findings to human carcinogenesis.

1. Rodriguez et al. (1997) Cancer Research 57, 2394.