51

Detection of NQO1 (NAD(P)H:Quinone oxidoreductase, EC 1.6.99.2) C609T polymorphism in archived human tumour tissue using PCR-RFLP

R.M. Phillips[1], S. Basu[1], P.M. Loadman[1], R. Puri[2], G.M. Flannigan[2], T. Shah[2], S. Martin[1], J.E. Brown[3], B. Naylor[4]. [1]*University of Bradford, Cancer Research Unit, Tom Connors Cancer Research Centre;* [2]*Bradford Royal Infirmary, Department of Urology, Bradford, United Kingdom;* [3]*University of Bradford, School of Pharmacy, Bradford, United Kingdom;* [4]*Bradford Royal Infirmary, Department of Pathology, Bradford, United Kingdom*

NQO1 is a cytosolic flavoprotein whose physiological function is believed to be the detoxification of quinone based xenobiotics. Paradoxically however, NQO1 also plays a central role in the activation of quinone based bioreductive drugs. The gene encoding for NQO1 is polymorphic (C to T transition at position 609 of human cDNA). Cells which are homozygous for the polymorphic variant are devoid of NQO1 enzyme activity leading to suggestions that individuals may be either predisposed to cancer (as a result of loss of a detoxification enzyme) or be resistant to quinone based anti-cancer therapies such as Mitomycin C. The C609T polymorphism can be detected by PCR-RFLP techniques designed to exploit the fact that the C to T base pair mutation introduces a Hinf 1 restriction site. Whilst current methods are successful using genomic DNA isolated from fresh tissues, the generation of 'clean' PCR products from DNA isolated from archival formalin fixed human tumour tissue is unsatisfactory. In this study, genomic DNA was isolated from 12 archived paraffin embedded human bladder tumour tissue (following approval by local research and ethics committee) and subjected to two rounds of PCR amplification using a nested primer strategy. In the first round of PCR, a 334 bp product was amplified following 35 cycles (94C: 1 min, 60C: 30s, 72C:45s) using forward (GAGACGCTAGCTCTGAACTGAT) and reverse (CTGCCTGGAAGTTTGGTCA) primers. In the second round of PCR using forward (ATTTGAATTCGGGCGTCTGCTG) and reverse (TCTAGTGTGCCTGAGGCCTCC) primers and 30 cycles of PCR (94C:1min, 63C:30s and 72C:30s), a clean PCR product of 217bp was obtained for all specimens tested. Digestion of this PCR product with Hinf 1 generated fragments of 217bp (wild type), 217bp, 161bp and 56bp (heterozygotes) and 161bp and 56bp (homozygous mutant) following separation on a 3% agarose gel. The method provides reliable and reproducible information on the NQO1 genotype status of genomic DNA isolated from archived formalin fixed, paraffin embedded human tissues. This method together with the use of archival tumour material should enable the design of retrospective studies to critically assess the role of the NQO1 polymorphic genotype in predicting tumour response to quinone based agents such as mitomycin C.

52

DT diaphorase and cytochrome P450 reductase protein localisation in archived bladder tumour specimens: correlation with clinical response following mitomycin therapy

S. Basu[1], J.E. Brown[2], R. Puri[3], T. Shah[3], G.M. Flannigan[3], B. Naylor[4], S. Martin[1], P.M. Loadman[1], J. Gill[1], R.M. Phillips[1]. [1]*University of Bradford, Cancer Research Unit, Bradford, United Kingdom;* [2]*University of Bradford, School of Pharmacy, Bradford, United Kingdom;* [3]*Bradford Royal Infirmary, Department of Urology, Bradford, United Kingdom;* [4]*Bradford Royal Infirmary, Department of Pathology, Bradford, United Kingdom*

Mitomycin C (MMC) is an adjuvant intravesical therapy used to prevent recurrences of superficial bladder tumours. Many enzymes have been implicated in the metabolic activation of MMC particularly DT Diaphorase (DTD) and Cytochrome P450 reductase (P450R). Recent studies have shown that response of human bladder tumour histocultures to MMC can be predicted based on analysis of DTD and P450R gene expression (Gan et al, Clin Cancer Res 7; 1313, 2001). The role of DTD in the activation of MMC is however controversial and the aim of this study therefore is to determine whether or not clinical response to MMC therapy can be predicted based on immunohistochemical analysis of DTD and P450R protein. In this retrospective study, 91 formalin fixed, paraffin embedded transitional cell carcinomas (TCC) of the bladder (from patients diagnosed more than 3 years ago) were collected following local research and ethical committee approval. Tumours used were representative of all grades (G1 [n=25]; G2 [n=42]; G3 [n=23]) of both superficial (pTa [n=40]; pT1 [n=32]; Cis [n=1]) and muscle-invasive (pT2 [n=18]) stages of TCC. Tissue arrays were constructed, sectioned and stained with antibodies for human DTD and P450R.The intensity of staining was scored (by 3 independent observers) using a scale of no stain, moderate stain and heavy stain. An inverse relationship exists between the stage of tumour and intensity of staining for DTD with 73% (n= 40) and 39% (n = 19) of Ta and T2 specimens recorded as positive for DTD respectively. In contrast, DTD staining intensity was independent of tumour grade. In the case of P450R, staining intensity increased with both tumour grade and stage although the majority of tumours were scored as moderate for expression of P450R protein. These findings are consistent with previous studies based on analysis of gene expression patterns and demonstrate that superficial TCC of the bladder have elevated levels of DTD. Clinical response criteria employed were time to first recurrence following treatment with single dose MMC post transuretheral resection. A broad spectrum of responses was observed (ranging from 2 to 36 months). Clinical response was independent of both DTD and P450R levels in tumours. Specific examples exist of poor response (time to recurrence = 3 months) with high DTD (moderate P450R). This study suggests that whilst DTD is a target for new drug development, prediction of response to MMC on the basis of DTD and P450R may be premature.

53

Product development of fenretinide, NSC 374551, intravenous formulation

S. Gupta[1], J. Zgodinski[2], R. Vishnuvajjala[1], D. Solomon[2]. [1]*National Cancer Institute, Developmental Therapeutics Program, Pharmacerutica;* [2]*Ben Venue Labs, Product and Process Development, Bedford, USA*

Purpose: It is not possible to prepare an injectable formulation of fenretinide owing to its poor aqueous solubility. The objective of these studies was to study the solubility profile of fenretinide in various solvents leading up to an intravenous formulation suitable for human clinical trials.
Methods: Diluent 12 is a mixture of equal volume of absolute alcohol and Cremophor EL, polyoxyethoxylated castor oil, USP. The drug solubility was studied in alcohol as a function of cremophor concentration. The concentrated solutions of the drug were prepared in alcohol/cremophor EL and diluted in NS and D5W at various dilution factors. The short term and long term stability of the concentrates and diluted solutions were followed. We also developed a lyophilized formulation of fenretinide and studied its long-term stability.
Results: The drug can be easily dissolved in diluent 12 at a concentration of 10 - 15 mg/mL. The drug solutions are stable at refrigerated and room temperature for up to 3 months with no significant loss in potency or build up of impurities. The Diluent 12 formulations can be conveniently diluted in NS or D5W to yield 1 mg/mL solution, which is stable for 4 days with no loss in potency of the solution. It is also possible to lyophilize the fenretinide formulations from t-butanol/water solutions. The concentration of t-butanol can be varied from 5% to 95%. The resulting amorphous powder can be reconstituted using diluent 12 and further diluted in normally used infusion solutions such as normal saline and 5% dextrose. The lyophilized powder is stable at room temperature.
Conclusions: A stable, lyophilized and ready-to-use liquid formulation of a fenretinide was developed.

Antimetabolites

54

Gene expression profiling of colorectal cancer cell lines exposed to 5-fluorouracil (5-FU) and 5-FdUrd

S. Easdale[1], P. Clarke[1], J. Titley[1], N. Cattini[2], R. Wooster[2], P. Workman[1]. [1]*Institute of Cancer Research, Cancer therapeutics, London, United Kingdom;* [2]*Institute of Cancer Research, Molecular Carcinogenesis, London, United Kingdom*

Although, 5FU is the mainstay of treatment for colorectal cancer, its precise molecular mechanism of action is undefined. It is already known that 5FU treatment results in inhibition of thymidylate synthase and misincorporation of its metabolites into both DNA and RNA. Microarray technology allows for the measurement of global gene expression and is poised to revolutionise the discovery and use of new anticancer agents. This project involved the analysis of gene expression patterns in three human colorectal cancer cell lines exposed to 5FU and one of its important metabolites 5FdUrd. The complex mechanisms of action of 5FU were expected to challenge the microarray technology. The three cell lines were exposed to five times their IC_{50} concentrations of 5FU or 5FdUrd and cell cycle distribution analysed over 2-72 hours. Messenger RNA was also extracted from cells exposed to the same drug concentrations and time course. In addition one of these cell lines, HCT116 was exposed to an increased dose of 5-FU, 375uM.

The mRNA was labelled with fluorescent dyes (Cy3 and Cy5) using reverse transcription and hybridised to 5808 cDNA clones arrayed on glass slides. The array slides were prepared in-house and encompass known genes and ESTs known or suspected to be associated with cancer, for example apoptosis genes and cell cycle genes. Cell cycle analysis showed that each cell line was arrested in the G1 phase of the cell cycle and a depletion of cells in S phase was also found. Each cell line was affected to a varying degree. Both 5FU and 5FdUrd treatments cause similar effects upon the cell cycle in these three cell lines. Microarray analysis of 5FU and 5FdUrd treated cells demonstrated that each cell line had its own distinctive pattern of gene expression, which was time and drug dependent. A considerable number of genes exhibited altered expression, either increased or decreased relative to control levels. In excess of 500 genes were altered over the entire course of the experiment within each cell line. No evidence of alteration of genes involved in pyrimidine metabolism or DNA/RNA metabolism was found. No evidence for p53 induced gene expression could be found in wild-type p53 HCT116 cells, in contrast to published data. Within one cell line, a number of integrin related genes appear to be affected by both 5-FU and 5-FdUrd. Whereas another cluster of genes affected within this cell line appear to relate to 5FU mechanism of action compared to 5FdUrd.

55

Combination of troxacitabine (troxatyltm) and cytosine arabinoside is beneficial in human leukemia cells

H. Gourdeau[1], J. Jolivet[1], L. Leblond[1], B. Hamelin[1], F. Ouellet[1], S. Barbeau[1], D. Bouffard[1]. [1]*Shire BioChem Inc., Cancer Biology, Laval, Canada*

Troxacitabine (Troxatyltm) is a novel β-L-nucleoside analogue with potent antineoplastic activity both *in vitro* and in several tumor models *in vivo*, and is presently in Phase II clinical trials. Recent results have shown that the combination of troxacitabine and araC in patients with leukemia has resulted in complete responses. To determine the mechanistic basis of this interaction, we have investigated, in the laboratory, the *in vitro* and *in vivo* effect of the combination of araC and troxacitabine against lymphoblastic CCRF-CEM leukemia. We observed that combination of troxacitabine and araC *in vitro* was synergistic with combination indices between 0.1 and 0.7 as calculated with the CalcuSyn software (Biosoft). The combination of troxacitabine and araC had a greater impact on DNA synthesis recovery than either agent alone. Analysis of the effect of troxacitabine on the intracellular metabolites of araC revealed that troxacitabine did not inhibit araC deamination and caused a slight decrease in the overall intracellular accumulation of araCTP. The lower accumulation of araCTP could not be attributed to the feedback inhibition caused by troxacitabine triphosphate on dCK. Furthermore, our *in vivo* experiments demonstrate that combination of araC and troxacitabine is better at slowing down the progression of leukemia in mice than either agent alone without additive toxicities. Injections i.p. qdx5 of 10mg/kg araC in combination with troxacitabine at 5 or 10 mg/kg increased ILS of mice to 43 and 57% compared to 34% for araC alone and 41 and 44%, respectively for troxacitabine alone: this represents an improvement in ILS of 8 and 17%, respectively when compared to araC alone at 10mg/kg. A pharmacokinetic study revealed that troxacitabine did not influence the disposition of araC. Overall, the results show that the anti-leukemic activity of troxacitabine and araC is complementary when both nucleoside analogues are used in combination. These effects appear to be related, at least in part, to their respective cytotoxic characteristics rather than to a pharmacokinetic interaction. These results encourage the use of troxacitabine and araC in combination as a first-line regimen for treatment of leukemia.

56

TP53 status may predict pathological complete remission (pCR) to cisplatin + fluorouracil + leucovorin (PFL) in ethmoidal intestinal type adenocarcinoma (ITAC) treated with preoperative chemotherapy

L. Licitra[1], F. Perrone[2], S. Suardi[2], M. Oggionni[2], S. Tabano[2], L.D. Locati[1], P. Bossi[1], G. Cantù[3], P. Quattrone[4], S. Pilotti[2]. [1]*Istituto Tumori Milano, Medical Oncology, Head & Neck Department;* [2]*Istituto Tumori Milano, Experimental Pathology Unit, Milan, Italy;* [3]*Istituto Tumori Milano, Surgery, Head and Neck Department;* [4]*Istituto Tumori Milano, Pathology Department, Milan, Italy*

Background: Intestinal-type adenocarcinoma (ITAC) of the ethmoid sinus is an uncommon tumor. The prognostic value of pCR is well recognized in H&N cancer. The pCR is defined as the absence of any tumor cell at pathological evaluation of the complete surgical specimen. A correlation between TP53 status and pathologic complete remission (pCR) to primary PFL chemotherapy followed by cranio-facial resection was performed within a prospective phase II trial on ethmoid ITAC. Of 37 ITACs included in the study 23 diagnostic specimen biopsies were available and analyzed for TP53 status. Chemotherapy consisted of PFL every 3 weeks for 2-5 courses. Pts not achieving a clinical partial remission after two cycles underwent immediate surgery.

Methods: Genomic DNA was extracted from microdissected tissue obtained from formalin-fixed, paraffin-embedded tissue. TP53 gene from exon 5 to exon 8 of all samples were amplified by PCR and screened by dg-DGGE (double gradient-Denaturing Gradient Gel Electrophoresis) analysis. In cases showing an abnormal dg-DGGE pattern an automated DNA sequencing was performed.

Results: Tumor extension (UICC 97), TP53 status mutated vs wild type, number of chemotherapy cycles and number of pathological complete remission are reported in the cross-table.

N. cycles	T1/T2		T3/T4		Total pts
	TP53mut	TP53wt	TP53mut	TP53wt	
≤ 2	–	1*	5	–	6
3-5	3	2	8	4	17
pCRs	–	2	-	4	6

*this patient received only 1 cycle of PFL for cardiac toxicity and was not in pCR at surgery

Conclusions: Preliminary results indicate that pCR to PFL is strongly related ($p<0.0001$) to TP53 status. TP53 status seems to predict response better than T extension. Number of total cycles seems not to play a role in obtaining a pCR in TP53 mutated tumors. In the future, better selection of potentially responsive patients will allow more individualised treatment approach. Supported in part by AIRC.

57

Rapid and complete thymidylate synthase (TS) inhibition in tumors after fluorouracil (5-FU) by methylene-tetrahydrofolate (ch2fh4) preloading

C.P. Spears[1], B. Gustavsson[2], G. Carlsson[2], E. Odin[2]. [1]*Sierra Hematology & Oncology, Sacramento, USA;* [2]*Sahlgrenska Institute, Surgery, Gothenburg, Sweden*

TS is inhibited by the metabolite of 5-FU, 5-fluorodeoxyuridylate (FdUMP), in formation of a covalent ternary complex with 5,10-methylene-tetrahydrofolate (CH2FH4), that is required in great excess for stable inhibition. High degrees of TS inhibition are needed for effecting thymidine depletion and cytotoxities. Leucovorin (LV) has been used as a folate source in attempts to expand tumor CH2FH4 pools and increasing TS inhibition. We have previously shown that CH2FH4 is stable as a pharmaceutical, with promising results in Phase I-II trial with weekly 5-FU (Cancer J 10:266-297, 1997). We now show that preloading by i.v. bolus administration of pharmaceutical CH2FH4 (50-200 mg total dose) before bolus 5-FU results in immediate and substantial expansion of tumor CH2FH4 levels which are associated with unprecedented degrees of immediate TS inhibition. Serial surgical tumor biopsies were obtained in 17 patients given CH2FH4, in 19 patients given LV (500 mg/sq m), and in 9 patients given 5-methyl-tetrahydrofolate (CH3FH4). Tumoral TS activities CH2FH4 levels were measured by [3H-FdUMP] binding assays. The predominant tumor type was colorectal cancer in all groups. In the LV group, TS levels were over 0.5 pmol/g at 20 min after 5-FU in 8 of the 19 patients, with average (± SD) TS inhibition only 67.1 ± 32.0 percent, and only 5 patients showed over 97% inhibition. Results with CH3FH4 were even less effective than LV. In contrast, tumors of patients receiving CH2FH4 showed over 97% inhibition in 14 of the 17 patients, and all but one had less than 0.5 pmol/g TS activity by 20 min after 5-FU. Levels of tumor folates showed selective increases in CH2FH4, and in CH2FH4/FH4 ratios, by more than two-fold, to an average of 0.50 nmol/g and to 36.3%, respectively. These results provide direct evidence that CH2FH4 is rapidly transported into tumor tissue as the parent molecule, for effecting profound TS inhibition by FdUMP. Supported in part by the Swedish Cancer Society and NCI CA39629.