AN INVESTIGATION OF THE ROLE OF THYMIDINE PHOSPHORYLASE IN THE ACTIVATION OF 5-FLUOROURACIL IN COLON TUMOUR CELL LINES

By

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ABSTRACT

Thymidine phosphorylase (TP) is identical to platelet-derived endothelial cell growth factor (PD-ECGF) and was originally isolated from platelets as the sole moiety with endothelial cell mitogenic activity. TP activity is increased in a number of tumour types compared with adjacent normal tissues including colon, stomach and breast cancer. TP has a role in tumour angiogenesis and increased activity in tumours confers a more aggressive tumour phenotype. TP also has a role in the activation of the fluoropyrimidine 5-fluorouracil (5-FU) to 5-fluorodeoxyuridine (5FdUrd). Intratumoural expression of TP is heterogeneous and may explain why only a proportion of patients respond to therapy with 5-FU. Modulation of 5-FU with interferon-α (IFN-α) increases the cytotoxicity of 5-FU in colon cancer cell lines. This is thought to occur through an increase in the activity of TP leading to greater formation of 5'-fluorodeoxyuridine monophosphate (FdUMP) and increased inhibition of thymidylate synthase (TS). HT-29 colon adenocarcinoma cell lines were transfected with TP cDNA and a novel TP activity assay demonstrated a 5-fold increase in TP activity in HT-29(TP) cells compared with the parental cells and the vector alone transfected controls, HT-29(V). 5-FU cytotoxicity was increased 1.6-fold in HT-29(TP) cells compared with HT-29(V) cells ($p<0.001$, Students' t-test). There was no significant difference in cytotoxicity between HT-29(TP) and HT-29 parental cells ($p>0.1$, Students' t-test). Transfection alone without TP cDNA increased the resistance of HT-29 cells to 5-FU. The apparent increase in 5-FU cytotoxicity in HT-29(TP) cells was not enhanced by Leucovorin (LCV) or Deoxyinosine and cytotoxicity was not significantly reversed by exogenous thymidine, although a small increase in 5-FU IC_{50} was observed in the controls. Inhibition of TS by FdUMP was observed in these cells, however it does not appear to be the critical mechanism of action of 5-FU. The data from this thesis suggest that an increase in the

activity of TP alone (5-fold) is not sufficient to significantly increase 5-FU cytotoxicity. Confirmation was obtained when TP activity and 5-FU cytotoxicity were measured in a number of cell lines from several tumour types. TP activity alone did not determine the sensitivity of the cell lines to 5-FU. Despite disappointing results with respect to 5-FU, a greater and more significant effect may be observed with 5-FU pro-drugs such as 5-dFUrd and Tegafur, which require activation by TP. The application of TP as a candidate gene for gene-directed/fluoropyrimidine pro-drug therapy is therefore promising as a means of improving the current lack of effective treatment for colon cancer and requires further investigation.

DEDICATION

To my husband, Tony

DECLARATION

The work in this thesis was performed personally unless otherwise acknowledged.

Lesley H. Milne
December, 1998

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5-FU	5-fluorouracil
5FdUrd	5-fluorodeoxyuridine
5FdUMP	5-fluorodeoxyuridine monophosphate
5FdUTP	5-fluorodeoxyuridine triphosphate
5FUrd	5-fluorouridine
5FUMP	5-fluorouridine monophosphate
5FUTP	5-fluorouridine triphosphate
TP	thymidine phosphorylase
TS	thymidylate synthase
IFN	interferon
LCV	Leucovorin
EDTA	diaminoethanetetra-acetic acid
SDS	sodium dodecyl sulphate
ECL	enhanced chemiluminescence
HRP	horseradish peroxidase
PMSF	phenylmethylsulfonylfluoride
PBS	phosphate buffered saline
DMSO	dimethyl sulphoxide

CHAPTER 1

1. Introduction

1.1 Recent Cancer Statistics

Over recent years there have been reductions in mortality from some cancers including Hodgkins's disease, Burkitt's lymphoma and testicular cancer. The American Cancer Society reports that since 1960 the death rate from cancer in children has decreased 62 percent (Rennie and Rusting, 1996). The three leading causes of cancer death are lung, breast and colorectal cancers and mortality from these cancers has started to decrease. Lung cancer mortality rates decreased by 3% between 1990 and 1992, partly due to a decrease in smoking. This disease is largely preventable. Mortality rates in women with breast cancer decreased by 5% between 1989 and 1993, stemming from improvements in treatment available as well as early detection. Finally, death from colorectal cancer fell by 17% between 1973 and 1992 again influenced by early detection. Colon cancer is however relatively resistant to treatment with systemic chemotherapy. The fluoropyrimidine 5-fluorouracil (5-FU) is the mainstay of treatment and improvement of the efficacy of this agent is an ongoing concern.

1.2 Colon cancer

Cancer of the colon is thought to develop through the acquisition of a sequence of genetic mutations (Boland, 1993). The primary carcinogens responsible for these mutations are not known. There are many carcinogens present in food and mutagenic compounds have been identified in the stool. These compounds are however, found not only in patients with colon cancer but also in control subjects.

Carcinogenesis of the colon can be divided into a series of pathological stages. In the normal colon, proliferation occurs in the lower two thirds of the colonic crypt.

Cells migrate up the crypt and upon arrival at the luminal surface they differentiate in order to function. At a later stage apoptosis occurs and the cells are sloughed into the lumen. A balance is therefore present between the generation of new cells, migration up the crypt and the subsequent loss of differentiated cells. Cells at the base of the colonic crypt are protected from damage from environmental carcinogens. The differentiated cells, however, are exposed to these carcinogens and it is vital that programmed cell death occurs in these cells in order that damage may not be replicated.

The initial stage of carcinogenesis occurs when there is a shift in the zone of proliferation to the upper third of the colonic crypt. This results in a failure of the proliferating epithelial cells to differentiate and then apoptose. Cells accumulate and form an adenomatous polyp which constitutes the second stage of neoplastic development. Interestingly, cells in the first two stages of neoplasia have the ability to spontaneously regress. They are benign since they do not have the ability to invade or metastasise. They can, however, grow and develop higher grades of epithelial dysplasia. Mutations in the Ki-*ras* proto-oncogene may play a role in this development although not all colorectal neoplasms show this lesion (Vogelstein, 1988). It is hypothesised that the tumour becomes malignant, as a result of the accumulation of mutations and other genetic instabilities which do not allow cell death to occur. Instead the cell continues to proliferate thus creating greater genetic instability, such as chromosome deletions, duplications and rearrangements. In particular, in 75% of colon tumours one copy of the gene for p53 (on chromosome 17p) has been lost and in most cases the remaining allele is mutated (Vogelstein, 1989). If the function of the p53 gene is lost, there is no cell cycle arrest in which repair of DNA damage can occur and less programmed cell death, leading ultimately to greater loss of heterozygosity and genetic instability. When the normal copy of p53 is restored, the cells lose their tumourigenic properties (Baker, 1990).

A deletion on chromosome 18q has also been demonstrated in 80% of colorectal tumours resulting in the loss of another tumour suppressor gene (Fearon, 1990). The resultant loss of heterozygosity is reported only in malignant lesions and is thought to be of importance in the progression from a benign lesion to malignant neoplastic tissue.

In time, a fraction of cells develop a combination of genetic instabilities which gives them the ability to digest the extracellular matrix, invade through adjacent tissues, infiltrate the bloodstream and create metastatic tumours at sites distant from the primary tumour. This metastatic disease is the main cause of cancer death in patients.

The main risk factor associated with colorectal cancer is a family history of colon cancer, polyps or inflammatory bowel disease. Exposure to pollutants through living in industrial or urban areas and high fat and low fibre diets are also thought to be contributory factors (Boland, 1993).

Much is known, therefore, about the biology of this disease in terms of the various stages of carcinogenesis. Colon cancer is however, relatively resistant to treatment with systemic chemotherapy. 5-FU has been the mainstay of treatment since the mid-1950's. However, the efficacy of this agent, is limited in a substantial number of patients with a response rate of 10-20% (defined as a 50% reduction in tumour dimensions) as a single agent for standard bolus administration (Grem and Fischer, 1989, Sotos, 1994 and Abbruzzese, 1989).

Most patients with colon cancer present with disease limited to a local site although some present with unresectable or metastatic disease. Those with resectable disease will be treated with surgery to remove all visible disease and are thus potentially curable at this stage (August, 1984). In certain cases surgery is combined with chemotherapy (Rennie, 1986). Despite surgery many patients relapse months or years later due to the presence of occult metastases which were present but undetected at surgery (Saltz, 1997). Adjuvant chemotherapy based on 5-FU reduces the risk of relapse

in certain groups of patients. Treatment for advanced or metastatic disease is usually palliative rather than curative and based around chemotherapy, again with 5-FU as the single most important agent.

1.3 5-Fluorouracil

The 5-fluorinated pyrimidines were designed and synthesised following the observation that rat hepatomas utilised radiolabeled uracil to a greater degree than normal liver (Rutman, 1954; Heidelberger, 1957). This suggested that the enzymatic pathways for the utilisation of uracil were upregulated in tumour cells compared to normal tissues and therefore could be a potential target for chemotherapeutic agents.

5-FU is an analogue of uracil with a fluorine molecule substituted at the carbon 5 position of the pyrimidine ring in place of hydrogen (Figure 1.1). It is used in the treatment of solid tumours such as breast, head and neck and gastrointestinal cancers. In order to exert it's cytotoxic effects, 5-FU requires anabolism to cytotoxic nucleotides. Despite the fact that fluorine is larger than hydrogen, this does not hinder it's anabolism to nucleotides.

5-FU rapidly enters the cell via the same facilitated transport mechanism as uracil, adenine and hypoxanthine (Domin, 1993). Phosphorylation of 5-FU rather than intracellular entry, appears to be the rate-limiting factor in the formation of cytotoxic nucleotides (Wohlhueter, 1980 and Bowen, 1979).

1.3.1 Anabolism of 5-FU

The cellular anabolism of 5-FU and the interaction of it's active products with intracellular targets have been intensively investigated. Three pathways of anabolism have been identified (Figure 1.1).

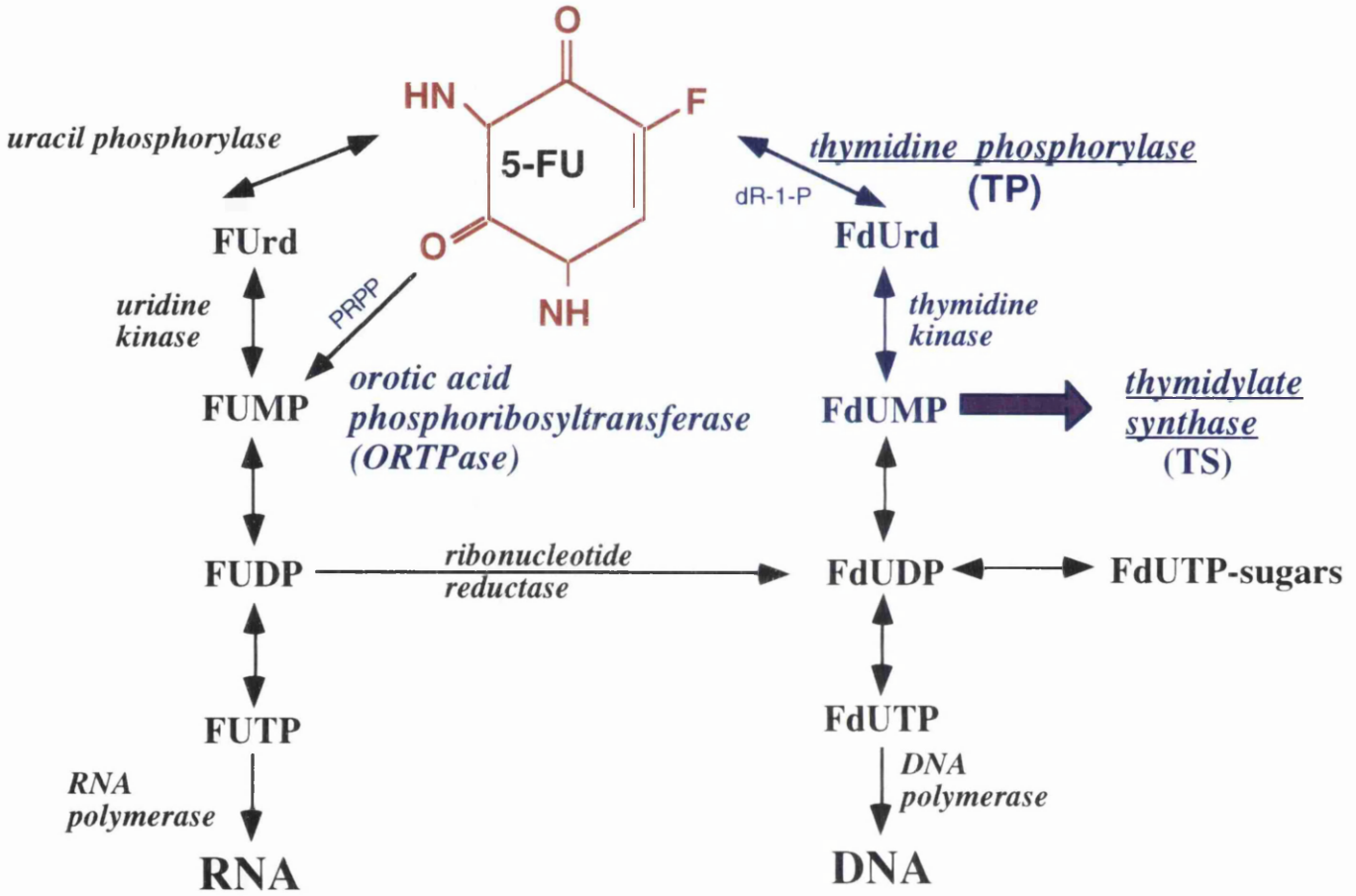


Figure 1.1 5-FU anabolic pathways.

Firstly, 5-FU can be converted to fluorodeoxyuridine (FdUrd) in the presence of deoxyribose-1-phosphate (dR-1-P) by thymidine phosphorylase (TP) followed by phosphorylation to fluorodeoxyuridine monophosphate (5-FdUMP) by thymidine kinase. 5-FdUMP can also be formed indirectly as a consequence of the conversion of fluorouridine diphosphate (FUDP) by ribonucleotide reductase to FdUDP and subsequent dephosphorylation. The cytotoxic nucleotide 5-FdUMP inhibits thymidylate synthase. Secondly, 5-FdUMP can be further phosphorylated by pyrimidine monophosphate kinase to FdUDP and then by pyrimidine diphosphate kinase to the cytotoxic nucleotide FdUTP. This nucleotide can compete with dUTP for DNA polymerase and become incorporated into DNA. Finally, 5-FU can be anabolised to fluorouridine monophosphate (FUMP) either through direct conversion by orotic acid phosphoribosyltransferase (ORTPase) in the presence of 5-phosphoribosyl-1-pyrophosphate (PRPP) or through the sequential action of uridine phosphorylase (forming fluorouridine, FdUrd) and uridine kinase. FUMP may be further phosphorylated to the cytotoxic nucleotide fluorouridine triphosphate (FUTP) which can compete with UTP for RNA polymerase and become incorporated into RNA.

1.3.2 Catabolism of 5-FU and it's nucleotide derivatives

In humans, approximately 90% of administered 5-FU is degraded through a catabolic pathway (Heggie, 1987). The pyrimidine ring is firstly reduced by dihydropyrimidine dehydrogenase (DPD) as shown in Figure 1.2, to form the dihydropyrimidine dihydrofluorouracil (DHFU) in the presence of nicotinamide adenine dinucleotide phosphate (NADPH). This is the first and rate-limiting step in the catabolism of 5-FU which regulates the availability of 5-FU for anabolism.

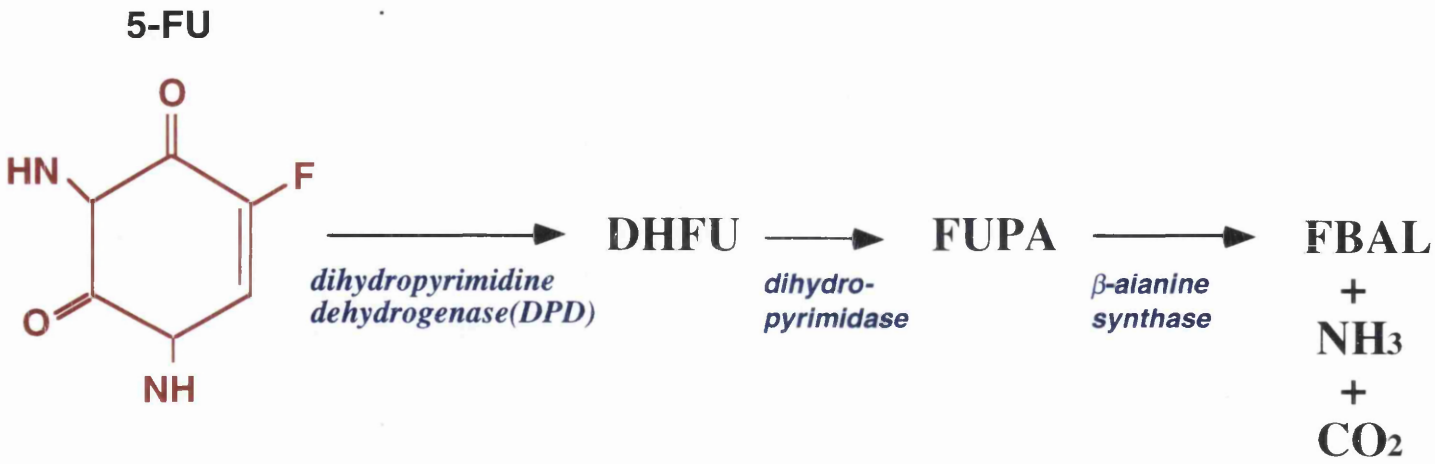


Figure 1.2 Catabolism Pathway of 5-Fluorouracil. The pyrimidine ring of 5-FU is reduced by dihydropyrimidine dehydrogenase (DPD) in the presesnce of nicotinamide adenine dinucleotide phosphate (NADPH) to form dihydrofluorouracil (DHFU). Dihydropyrimidase converts DHFU to α-fluoroureidopropionic acid (FUPA) which is then irreversibley converted to fluoro-β-alanine (FBAL), ammonia (NH_3) and carbon dioxide ($C0_2$).

Dihydropyrimidase converts DHFU to α-fluoroureidopropionic acid (FUPA) which is then irreversibly converted to fluoro-β-alanine (FBAL) ammonia and carbon dioxide by β-alanine synthase. FBAL and FUPA are excreted in the urine with FBAL being the principle urinary metabolite. Pharmacokinetic studies show that approximately 90% of the administered dose of 5-FU is eliminated by this pathway. Naguib *et al* (1985) measured DPD activity in several normal and neoplastic tissues and found the highest DPD activity in liver and lymphocytes in comparison to lower activities in the pancreas, lung and intestinal mucosa.

Catabolism of the nucleotide derivatives of 5-FU also occurs. Acid and alkaline phosphatases are capable of converting nucleotides to nucleosides. For example, 5-FdUMP can be converted to FdUrd by 5'-nucleotidases. In addition, the pyrimidine phosphorylases responsible for the phosphorylation of pyrimidine bases to nucleoside are reversible (Figure 1.1).

1.3.3 Modes of Action

Three known mechanisms of action of 5-FU have been elucidated at three different cellular targets: TS, RNA and DNA.

1.3.3.1 Inhibition of thymidylate synthase

One site of 5-FU cytotoxicity is the enzyme thymidylate synthase (TS), which is discussed in detail in section 1.4. TS catalyses the methylation of deoxyuridine monophosphate (dUMP) to thymidylate (dTMP) and is responsible for the only *de novo* source of dTMP. dTMP is the precursor for one of the four deoxyribonucleotides required for synthesis of DNA. The cytotoxic nucleotide 5-FdUMP competes with dUMP for the active site on TS (Figure 1.3). 5-FdUMP binds covalently to TS in the presence of reduced folates to form a covalent reversible ternary complex. This results

in the depletion of dTMP necessary for DNA synthesis. An accumulation of dUMP also occurs resulting in the formation of dUTP which may become incorporated into DNA.

1.3.3.2 RNA Cytotoxicity

Secondly, FUTP formed by the ribonucleotide pathway competes with UTP for RNA polymerase. FUTP is incorporated into both nuclear and cytoplasmic RNA altering it's processing and function in human colon cell lines in culture (Greenhalgh and Parish 1990). This has been shown to be the growth-limiting mechanism of action of 5-FU in MCF-7 breast carcinoma cells, where incorporation of 5-FU into total RNA correlates with loss of clonogenic survival (Kufe and Major, 1981). The specific mechanism whereby incorporation of 5-FU into RNA leads to cytotoxicity is unclear. A study of Friend erythroleukemia cells demonstrated that cytotoxicity related to incorporation into pre-ribosomal RNA resulting in an inhibition of maturation to cytoplasmic rRNA species (Herrick, 1984) and this has also been demonstrated in HT-29 colon carcinoma cells (Greenhalgh and Parish, 1990).

5-FU exposure may also result in changes in mRNA processing and translation, for example, polyadenylation of mRNA is inhibited by low concentrations of 5-FU resulting in increased instability (Carrico, 1979). Some specific species of mRNA and their respective proteins are altered following exposure to 5-FU. A dose-dependent increase in dihydrofolate reductase (DHFR) mRNA was reported in cells resistant to Methotrexate (Dolnick, 1985). In addition, the gene product had a reduced affinity for it's substrate. An increase in thymidylate synthase mRNA (one of the principle targets of 5-FU cytotoxicity) and a reduced affinity for it's substrate 5-FdUMP was observed in cells resistant to 5-FU (Washtein, 1983; Berger, 1984).

The ribonucleotide route of anabolism appears to be the principle pathway for anabolism of 5-FU in normal tissues since inhibition studies with a nucleotide

metabolite of allopurinol (which blocks 5-FU activation by OPRTase) demonstrated a reduction in toxicity to the gastrointestinal mucosa and bone marrow (Schwartz, 1979 and 1980, Houghton, 1980 and 1983).

1.3.3.3 DNA cytotoxicity

The third site of 5-FU cytotoxicity is DNA. FdUTP and dUTP compete with dTTP for DNA polymerase and subsequent incorporation into DNA. FdUTP incorporation into DNA contributes to 5-FU cytotoxicity in cell lines *in vitro* (Lonn, 1984 and 1986; Cheng, 1983; Major, 1982) and into marrow and tumour cell DNA *in vivo* (Sawyer, 1984). FdUrd exposure can result in the formation of large (1-5 megabase) DNA fragments as a result of double strand DNA breaks. The time course and extent of DNA megabase fragmentation was shown to correlate with loss of clonogenicity in HT-29 cells (Dusenbury, 1991). In addition, a depletion of thymidine monophosphate (dTMP), due to inhibition of TS by 5-FdUMP, results in the incorporation of dUTP into DNA in place of dTTP.

Two mechanisms prevent the incorporation of false nucleotides into DNA. dUTP hydrolase cleaves dUTP and FdUTP to dUMP and 5-FdUMP respectively (Ingraham, 1980 and Mauro, 1993). Uracil-DNA-glycosylase catalyses the hydrolysis of the 5-fluorouracil- and uracil-deoxyribose glycosyl bond of 5-FdUMP and dUMP residues present in DNA (Ingraham, 1980 and Mauro, 1993). Further endonucleolytic cleavage of the remaining deoxyribose sites results in a strand break which is subsequently repaired. Increased activity of dUTP hydrolase and Uracil DNA glycosylase contributes to resistance to 5-FU in KB cells (Cardonna and Cheng, 1980) and in resistant PEO4 ovarian cells (Chu, 1990a).

Alterations in DNA stability have been demonstrated following depletion of dTTP and FdUTP incorporation (Yin, 1991, Lonn, 1986 and Lonn 1988). 5-FU

treatment results in inhibition of DNA elongation and causes a reduction in the average DNA chain length (Schuetz, 1985). DNA strand breakage also takes place although the exact mechanism through which this occurs is unclear. The inability of cells to excise or repair nucleotides from DNA may contribute to strand breakage, since dTTP depletion means that there are insufficient amounts of nucleotides for replacement (Yoshioka, 1987). In turn, more dUTP may become falsely incorporated and so the process of excision and repair continues.

Factors downstream of damage also influence drug sensitivity e.g. p53 and *bcl-2*. The tumour suppressor gene p53 is known to play a vital role in the repair of DNA damage. Following DNA damage, expression and stability of p53 is induced (Kastan, 1991) and either cell cycle arrest occurs allowing repair of DNA damage (McIlwrath, 1994) or apoptosis occurs (Clark, 1993, Lowe, 1993). Normal p53 function can become disrupted by mutation of the gene, causing cells to continue proliferating despite DNA damage. Apoptosis is a key event in the killing of human tumour cells growing as xenografts in nude mice *in vivo* (Inada, 1997)

The means by which 5-FU exerts it's cytotoxic effects are many and varied. Which cytotoxic mechanism(s) predominates is thought to depend on the patterns of intracellular 5-FU metabolism which can vary among different normal tissues and tumour types and with drug concentration and duration of exposure.

1.4 TS Inhibition

It has long been recognised that inhibition of TS by 5-FdUMP is one of the principle mechanisms underlying 5-FU action. The kinetics of this reaction have been extensively studied (Danenberg, 1979). 5-FdUMP binds to a specific site on TS exposing a second site for the binding of 5,10-CH_2THF the reduced folate necessary for the stabilisation and retention of the ternary complex (Santi, 1987). TS inhibition is

therefore determined by a number of variables, including the levels of TS, the amount of 5-FdUMP present and it's rate of breakdown, the concentrations of dUMP which compete with 5-FdUMP and finally the levels of reduced folates and the extent of their polyglutamation (Figure 1.3).

The covalent ternary complex between 5-FdUMP and TS is reversible and persistence of the complex correlates with cytotoxicity. This is determined by the levels of free 5-FdUMP and reduced folates and also by the levels of dUMP. High levels of dUMP can aid in restoration of TS activity (Myers, 1975).

TS inhibition correlates with response to 5-FU in patients with colon and breast adenocarcinomas (Spears, 1984). Tumours from 3 patients who benefited from treatment with 5-FU demonstrated greater TS inhibition than the tumours from non-responding patients.

Overexpression of TS in tumours from patients with colorectal and gastric tumours correlates with resistance to 5-FU based therapies (Johnston, 1995) and this has also been demonstrated in cells in culture (Johnston, 1992). On the other hand, a retrospective study of rectal cancer patients demonstrated that increased TS levels at the time of surgery had prognostic significance (Johnston, 1994). Patients with Duke's B and C rectal cancer with high intrinsic TS levels derived the greatest benefit from adjuvant chemotherapy (Semustine, Vincristine (Oncovin®) and 5-FU, known as MOF). Adjuvant chemotherapy seemed to be of no added benefit in patients with low TS levels. The explanation for this unexpected result is uncertain. Patients with low TS levels may not have demonstrated survival benefit from chemotherapy because of the relatively non-vascularised nature of their tumours. In addition, the other drugs given in combination with 5-FU may have contributed to their response.

Alterations in the TS gene have been correlated with resistance to fluoropyrimidines, both intrinsic and acquired. Two point mutations have been reported

in a colon cell line insensitive to 5FdUrd (Barbour, 1990). This caused structural changes in TS leading to reduced affinity for both 5-FdUMP and 5,10-CH_2THF. Amplification of the TS gene resulting in increased enzyme levels has also been found in cell lines resistant to 5-FU and FdUrd (Berger, 1985 and Clark, 1987).

The inhibition of TS by 5-FdUMP can be reversed by exogenous thymidine, which is routinely used to determine the relative importance of this mechanism of action of 5-FU in studies *in vitro* (Murgo, 1980). Exogenous thymidine is not always successful in completely reversing 5-FU cytotoxicity. This suggests that in some cases other mechanisms exist alone or in addition to TS inhibition. For example, TS inhibition was the principle mechanism of action of 5-FU at lower concentrations (5-20μM) in mouse sarcoma 180 cells since thymidine reversed toxicity (Evans, 1980). At higher concentrations, however, thymidine was unable to reverse toxicity suggesting an alternative mechanism of cytotoxicity. Studies *in vivo* have demonstrated that when thymidine is administered with 5-FU incorporation into DNA is inhibited (Nayak, 1992). This also results in increased toxicity of 5-FU to normal tissues in various animal models by increasing incorporation into RNA (Speigelman, 1980 and Santelli, 1978).

1.5 TS Regulation

TS is therefore widely regarded as an important target for the anti-tumour activity of 5-FU. It's activity is more pronounced in tissues which are rapidly proliferating due to it's role in the synthesis of DNA (Conrad and Ruddle, 1972 and Johnson, 1984).

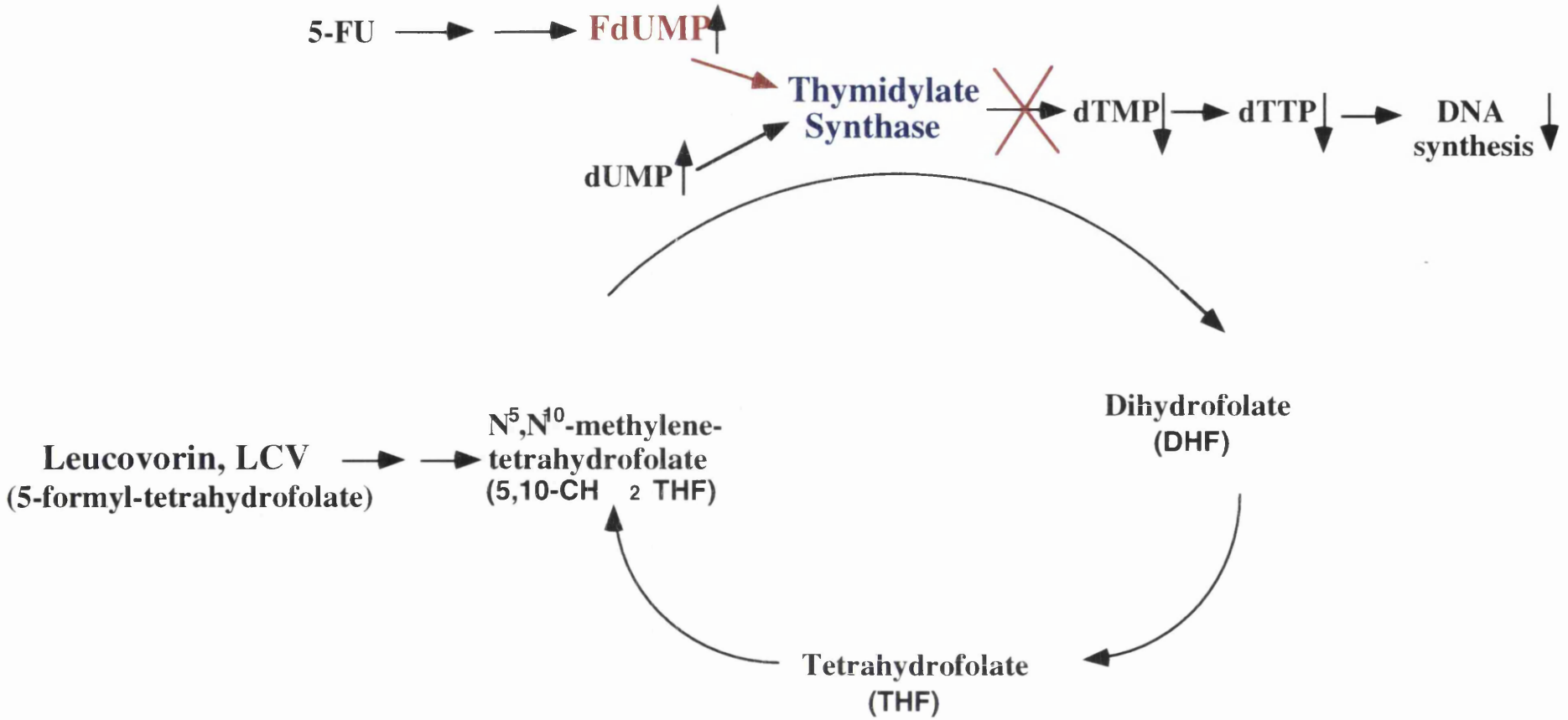


Figure 1.3 Thymidylate synthase catalyses the methylation of dUMP to thymidylate (dTMP) in the presence of the folate co-factor N5,N10-methylene tetrahydrofolate (5,10-CH_2 THF). FdUMP competes with dUMP for the active site on TS, leading to inhibition of the enzyme, accumulation of dUMP, FdUMP and depletion of dTMP, dTTP and consequently DNA synthesis. Exogenous Leucovorin is metabolised intracellularly, thus increasing the pools of reduced folates and increasing TS inhibition.

TS activity increases as cells enter S-phase of the cell cycle suggesting that TS levels are associated with cellular proliferation and DNA synthesis (Pestalozzi, 1995). In addition it's activity increases in response to 5-FU exposure, a resistance mechanism in tumour cells *in vitro* and *in vivo* that is discussed in more detail in chapter 4.

Regulation of TS occurs at the translational level with levels of mRNA remaining unchanged. TS mRNA translation is inhibited by the presence of TS protein (Chu, 1991). TS protein binds to specific regions on it's mRNA and mutations in these regions hinder TS binding (Chu, 1993).

1.6 Resistance

Resistance to 5-FU can occur as a result of alterations at many stages of the anabolic and catabolic pathways. For example, correlations between enzyme activity and sensitivity to 5-FU have been made for OPRTase (Piper and Fox, 1982), uridine phosphorylase (Peters, 1986) uridine and thymidine kinase (Bresnick, 1965 and Hande 1978) and TP (El-Assouli, 1985 and Piper, 1982). In addition, the availability of the co-factors for these activating enzymes is also of importance and may alter the anabolism and therefore response to fluoropyrimidines (Cory, 1979, Cadman, 1979 and Washtein, 1984). Increased activity of the target enzyme TS following exposure to 5-FU or elevated levels of the nucleotide dUMP can result in reduced 5-FU cytotoxicity. Depleted levels of reduced folates necessary for stabilisation of the inhibitory ternary complex with 5-FdUMP will also alter response to 5-FU. Finally, as discussed previously, increased activity of enzymes responsible for the excision and repair of DNA damage (Cardonna & Cheng, 1980) and the cellular response to DNA damage (e.g. p53) may reduce 5-FU cytotoxicity.

1.7 Modulation of 5-FU

5-FU is an attractive target for biochemical modulation due to the complexity of it's anabolic pathways and it's multiple sites of action. A number of strategies have been explored in order to increase the efficacy of 5-FU. These include attempts to enhance it's binding to TS, increase incorporation into DNA or RNA, decrease the catabolism of 5-FU and it's metabolites or increase the conversion of 5-FU to it's active metabolites.

1.7.1 Leucovorin and 5-FU

One such strategy to enhance the cytotoxicity of 5-FU has been the co-administration of Leucovorin (LCV). Leucovorin (5-formyl-H_4PteGlu, folinic acid) is a reduced folate which is metabolised intracellularly to 5,10-methylene-H_4PteGlu (Figure 1.3). This results in expansion of the intracellular reduced folate pools and permits maximal ternary complex formation with TS and the 5-FU metabolite 5-FdUMP (Wright, 1989).

The stability and increased retention of the ternary complex between 5-FdUMP, TS and 5,10-CH_2-FH_4 is an important determinant of 5-FU cytotoxicity. High concentrations of reduced folates are required for optimal binding and stabilisation of the ternary complex (Santi, 1974). Prolonged inhibition of TS results in greater depletion of dTTP, greater inhibition of DNA synthesis and ultimately an increased cytotoxicity (Pinedo, 1988). Resistance to 5-FU has been associated with insufficient concentrations of intracellular reduced folates (Houghton, 1981).

LCV is a chemically synthesised compound which must be metabolised intracellularly to exert it's effects on 5-FU (Boarman, 1992). It is transported across the cell membrane by a saturable reduced-folate carrier prior to metabolism and polyglutamation. Variations in the polyglutamation of reduced folates determine the extent of modulation by Leucovorin, therefore prolonged exposure to LCV is more

beneficial. Important aspects of resistance to modulation by LCV include impaired transport, metabolism and polyglutamation.

This combination has been extensively investigated in the clinic (Machover, 1986 and Poon, 1991). Phase III trials have shown an advantage of the combination in terms of response rate and time to disease progression (Poon, 1989 and Piedbois, 1992). A meta-analysis of nine randomised trials of 5-FU/LCV in patients with advanced colorectal cancer compared to 5-FU alone, demonstrated that 5-FU/LCV was significantly more beneficial than 5-FU alone in terms of tumour response rate (23% versus 11%, Piedbois, 1992). There was no advantage with respect to overall survival however. In the adjuvant setting, a trial of patients with Dukes stage B and C colon cancer demonstrated that 5-FU/LCV did result in a survival advantage over MOF (Wolmark, 1993).

1.7.2 Interferon and 5-FU

1.7.2.1 INF and 5-FU in colon cell lines in vitro

Interferons (IFNs) are involved in the regulation of a broad range of cell functions and modulate responses to infection and malignancy. IFN-α, -β and -γ bind to high affinity cell receptors activating a signalling pathway which leads to changes in gene expression, protein expression, enzyme activities, cell cycle distribution and nucleotide pools (Goldstein, 1986).

A number of studies in colon tumour cell lines have investigated the combination of 5-FU and IFNs. These studies have demonstrated that the IFNs can synergistically enhance the cytotoxicity of 5-FU in human (Wadler, 1990; Chu, 1990; Schwartz, 1992; Tevaearai, 1992; Eda, 1992; Houghton, 1993) and murine (Elias, 1988) cell lines.

The mechanism by which IFNs exert their effect on 5-FU cytotoxicity *in vitro* has been evaluated and a number of explanations given. IFN-γ abrogated the 5-FU induced increase in TS expression in H630 colon carcinoma cells, resulting in greater TS inhibition and 5-FU cytotoxicity (Chu, 1990). The interaction between 5-FU and IFN-α in these cells was at the level of DNA damage and the key factor appeared to be an increase in TP activity resulting in increased concentrations of 5-FdUMP via FdUrd, consequently greater TS inhibition and 5-FU cytotoxicity. Houghton and colleagues reported that the level of DNA single and double strand breaks were increased by IFN-α in GC_3/c1 colon adenocarcinoma cells (Houghton, 1993). There was further potentiation by the addition of exogenous LCV resulting from inhibition of TS and thymineless stress.

Schwartz demonstrated that IFN-α enhanced 5-FU cytotoxicity in HT-29 cells through an increased formation of 5-FdUMP (Schwartz, 1992). No change in the levels of 5-FdUMP, FUDP and FUTP was detected. There was no increase in intracellular 5-FU concentrations and no measurable change in incorporation of 5-FU into DNA or RNA, suggesting TS inhibition as the cytotoxic mechanism in these cells. In addition, an increase in the enzyme activity of thymidine phosphorylase, one of the first enzymes involved in the activation of 5-FU to cytotoxic nucleotides was demonstrated. These particular studies were the inspiration for the present study and are discussed in chapter 4. Schwartz later showed that this modulation could be further enhanced by the addition of the co-substrate for TP, dR-1-P, which appeared to be rate-limiting (Schwartz, 1994).

1.7.2.2 IFN and 5-FU, Clinical, Phase I

Of the three separate classes of IFNs (α, β, γ), IFN-α's have been most widely used clinically and the sub-type IFN-α2a has been most frequently utilised in clinical trials combined with 5-FU.

The early *in vitro* studies persuaded Wadler and colleagues to conduct a Phase I clinical study in patients with advanced colorectal cancer (Wadler, 1989). Patients were treated with 5-FU 750mg/m^2 i.v. continuous infusion daily for 5 days followed by a one week rest period, then weekly bolus therapy at 750mg/m^2 and IFN-α 9mU administered sub-cutaneously 3 times weekly starting on day 1. Thirty evaluable patients were included, 17 previously untreated with chemotherapy. Thirteen of the 17 previously untreated patients responded to the treatment regime. Toxicity was significant and one toxic death was reported. A clinical series update from 1990 (Wadler, 1990) demonstrated that of the 32 previously untreated patients there were 20 partial responses corresponding to a 63% response rate. Three toxic deaths occurred in the expanded series.

1.7.2.3 IFN and 5-FU, Clinical, Phase II

Following this promising study, a number of Phase II studies were conducted over the next few years. Twelve studies have been published over the period 1990-94 on a total of 548 patients 509 of whom were evaluable (Wrighely, 1992; Wadler, 1990; Pazdur, 1990; Weh, 1992; Rubio, 1992; Pazdur 1993; John, 1993; Finlay, 1994). A response rate of 28% was observed (142 recorded responses out of 509 patients) and 15 complete responses (3%). Eight toxic deaths (2%) were also reported. These studies were unable to confirm the impressive activity of this treatment regime originally reported by Wadler. A 28% response rate with 2% toxic deaths is no improvement from the results obtained with 5-FU and Leucovorin and appears more toxic.

1.7.2.4 IFN and 5-FU in vivo, phase III

A number of randomised Phase III studies were also initiated to confirm the results of Wadler and to investigate any impact on patient survival. As expected, the comparative arms in the studies used different 5-FU regimens. Four studies used the

Wadler schedule of 5-FU and IFN-α (York, 1993, The Corfu-A Study Group, 1995, Dofour, 1994 and Hill 1995). Two studies used a modified dose of 5-FU and/or IFN-α (Kohne, 1995 and Kreuser, 1995). One study combined 5-FU and IFN-α with hydroxyurea (Di Constanzo, 1995) and another used protracted i.v. 5-FU infusion over 10 weeks. There was also one double modulation study using both Leucovorin and IFN-α (Seymour, 1994).

A total of 1727 patients have been included in these Phase III studies. Only one study (Dufour, 1994) describes a significant difference in response between 5-FU alone and 5-FU and IFN-α (27% versus 10% for 5-FU alone). Survival was 12.3 months for the combination compared with 10.1 months for 5-FU alone. The remaining studies either demonstrate no difference (York, 1995, The Corfu-A Study group, 1995, Di Constanzo, 1995, Seymour, 1994, Hill, 1994 and 1995) or even a significant difference in favour of 5-FU without IFN (Kohne, 1995 and Kreuser, 1995). Most studies report significant increases in toxicities in the IFN-containing groups.

Results therefore suggest that there is no advantage in using the 5-FU/IFN-α combination together in place of 5-FU alone. In fact, the disadvantage of this regime is clearly the pronounced toxicity observed including abdominal pain, constipation, diarrhoea, fatigue, fever, influenza-like symptoms, nausea/vomiting, shivering, somnolence and stomatitis (Corfu-A Study Group, 1995). Focus has been especially on a toxicity syndrome characterised by watery diarrhoea followed by life threatening sepsis (Van Hoff, 1991).

1.7.2.5 IFN and 5-FU Summary

In summary, IFN-α potentiates the anti-tumour activity of 5-FU in colon cancer *in vitro*. A likely mechanism for this action is the induction of TP, resulting in increased formation of the nucleotide 5-FdUMP (presumably through the sequential actions of TP

and thymidine kinase on 5-FU) and greater inhibition of TS. This modulation can be further enhanced by the addition of the co-substrate for TP, dR-1-P. The results of clinical studies were initially very promising however have not been achievable in later studies. This combination gives no greater a response rate than 5-FU and Leucovorin, is costly, and toxic.

Since IFN-α acts, in part, through the elevation of TP activity, the current study will explore the hypothesis that colon cancer cells transfected with the gene for TP *in vitro* will be more sensitive to the effects of 5-FU. If so, this system could be exploited through the application of gene therapy techniques directing tissue specific expression of TP in a transient fashion, rendering tumour cells more sensitive to fluoropyrimidines such as 5-FU and avoiding the unnecessary side effects of INF-α.

1.8 Thymidine phosphorylase

The characteristic role for TP in cells is in thymidine metabolism and homeostasis. TP catalyses the reversible synthesis of thymidine and inorganic phosphate from thymine, using deoxyribose-1-phosphate as a co-substrate (Reaction (1), Figure 1.4). TP also catalyses a deoxyribosyltransfer from one deoxynucleotide to another base, to form a second deoxynucleoside (Reaction (2), Figure 1.4).

Reaction 1:	Thymidine + $Phosphate_i$	$\rightleftharpoons$	Thymine + dR-1-P
Reaction 2:	Thymine* + Thymidine	$\rightleftharpoons$	Thymidine* + Thymine

Figure 1.4 The enzymatic reactions of thymidine phosphorylase

TP is highly expressed in the liver, lung, spleen, lymph nodes and peripheral lymphocytes (Yoshimura, 1990). Western immunoblotting has revealed that TP consists of 2 identical sub-units; the molecular weight of each sub-unit is approximately 45kDa

in bacteria and 55kDa in mammals. Human TP has been reported to be identical to the angiogenic factor platelet-derived endothelial cell growth factor (PD-ECGF), both being products of the same gene (Moghaddam and Bicknell, 1992; Furukawa, 1992 and Sumizawa, 1993). Recombinant PD-ECGF has TP activity (Sumizawa, 1993). PD-ECGF was originally isolated as the sole moiety with endothelial cell mitogenic activity in platelets (Miyazono and Heldin, 1989). The TP activity of PD-ECGF promotes endothelial cell proliferation by reducing thymidine levels (Finnis, 1993) and is thought to be important in tumour angiogenesis in breast cancer (Fox, 1993; Miyazono, 1987 and Yonenga, 1998). It is a non-glycosylated intracellular protein which lacks a secretion signal peptide (Ishikawa, 1989 and Relf, 1997). PD-ECGF is also chemotactic for endothelial cells *in vitro.* There is a high expression of TP in macrophages in many tissues (Yoshimura, 1990 and Fox, 1995). Zimmerman and colleagues first reported that several human tumours, such as kidney, stomach and lung cancers have relatively high TP activity compared with normal tissues (Zimmerman, 1964). Yoshimura and colleagues also showed by Western immunoblotting that TP expression was higher in tumours of the stomach, colon and ovary, compared to the non-cancerous region of the same surgical sample (Yoshimura, 1990). Western blot analysis of TP in breast tumour tissue demonstrated overexpression in breast carcinomas compared to normal breast tissue, $p<0.001$ (Moghaddam, 1995). Therefore, these data are consistent with a role for TP in tumour proliferation. In addition, studies have shown that TP activity is elevated in the plasma of tumour bearing animals and in the plasma of patients with uncontrolled neoplastic disease compared to that of normal subjects (Pauly, 1977 & 1978).

Studies *in vitro* have shown that TP has angiogenic activity *in vivo* (Ishikawa, 1989) and *in vitro* (Moghaddam, 1995 and Miyadera, 1995). The catalytic activity of TP is essential for it's angiogenic activity, as shown using active site-directed mutants that maintained their secondary structure but had reduced TP activity (Moghaddam, 1995).

The mutants did not exhibit angiogenic activity in the rat sponge model. In addition, over-expression of TP by gene transfer in MCF-7 cells has no effect on breast carcinoma cell growth *in vitro*. It did however confer a growth advantage to these cells when grown as a xenograft in nude mice. This growth advantage could be as a result of an angiogenic advantage, although this remains to be proven.

In patients with breast cancer, a study of 100 invasive-ductal-carcinoma tissue samples, showed 39% to be positive for TP expression by immunohistochemistry (Toi, 1995). The expression was significantly associated with microvessel density, a marker for angiogenesis. This was also demonstrated in malignant ovarian tumours (Hata, 1998), endometrial cancer (Fijiwaki, 1998) and squamous cell carcinoma (Igarashi, 1998). This suggests that TP expression plays an important role in the neovascularisation of human breast cancer. In benign and malignant human TP expression also correlates with areas of high blood velocity (Toi, 1995 and Reynolds, 1994). The mechanism by which TP elicits growth of new blood vessels is as yet unknown. The realisation that PD-ECGF and TP are identical and that they have no classic growth-stimulatory activity indicates that they are indirectly angiogenic. It may be that modulation of nucleotide metabolism in the tumour environment could have a true effect on endothelial cell growth and angiogenesis.

1.9 Hypothesis

The hypothesis of this thesis is that the genetic transfer of the gene for TP will increase the sensitivity of colon cancer cells to the cytotoxic effects of the fluoropyrimidine 5-FU.

1.10 Objectives

The primary objective of this thesis was to gain a further understanding of the enzymes involved in 5-FU metabolism in colon cancer, in order that new therapies might be designed which would improve the therapeutic index of this agent. Recent literature concerning the modulatory effects of IFN-α on 5-FU highlights the potential importance of the enzyme TP in the activation of 5-FU. The definitive test would therefore be to genetically transfer the gene for TP into colon cancer cells *in vitro* and using cytotoxicity experiments to analyse the cells for increased sensitivity to 5-FU. This strategy could then be further evaluated as a potential gene therapy approach in human colon cancer patients.

The individual aims of the study were to characterise 5 colon carcinoma cell lines with respect to growth kinetics, sensitivity to 5-FU, protein expression of the enzymes TP and TS and TP activity. In addition, TP protein expression and activity would be measured in human colon tumour and normal biopsy pairs. Following characterisation of the cell lines, the aim was to transfect the cells with the gene for thymidine phosphorylase and assess mechanisms for increasing sensitivity to 5-FU.

Whilst these studies were being undertaken, Schwartz and colleagues carried out similar experiments in HT-29 colon adenocarcinoma cells (Schwartz, 1995). They showed that HT-29 cells transfected with TP had increased TP activity and were more sensitive to 5-FU. However in the same year a similar study in MCF-7 breast cancer cells failed to demonstrate a correlation between increased TP activity and sensitivity to 5-FU (Patterson, 1995). Because of the contradictory data, the present study went on to further clarify the role of TP in 5-FU cytotoxicity in colon cancer cells lines.

CHAPTER 2

2. Characterisation of Colon Cancer Cell Lines

2.1 Introduction

Colon cancer has been chosen as the model for these studies and a panel of colon adenocarcinoma cell lines were characterised. A number factors thought to influence the sensitivity of these cells to the chemotherapeutic agent 5-FU were also evaluated. This study was used as a starting point preceding the transfection studies.

Firstly, the growth kinetics of the cell lines was evaluated. Following sub-culture, cells progress through a characteristic pattern of growth consisting of a lag phase (an adaptation period), followed by a log phase (a period of exponential growth) and finally a stationary or plateau phase (confluence; growth fraction falls between 0 and 10%), (Freshney, 1987). These phases provide information about a cell line, such as the population doubling time. In addition, measurement of these phases of growth can be used to quantify the response of the cells to inhibitory or stimulatory culture conditions. For example variations in nutrient concentration, hormonal effects or toxic drugs. In the current study, the population doubling times of a panel of colon adenocarcinoma cell lines were measured.

Secondly, the expression of enzymes thought to be important in the activation and cytotoxicity of 5-FU was evaluated. As discussed earlier, TP is the first enzyme involved in a series of reactions resulting in the activation of 5-FU to 5-FdUMP, the cytotoxic nucleotide which inhibits TS, Figure 1.1. The expression of these two enzymes was therefore measured by Western Immunoblotting. Antibodies raised against TP and TS were made available by Dr Roy Bicknell and Prof. Patrick Johnston respectively.

Thirdly, following evaluation of the five colon cell lines for TP and TS protein expression, the cells were characterised for their sensitivity to 5-FU. There are a number of methods that can be employed for measuring the cytotoxic potential of anti-cancer drugs. Two such methods are the clonogenic assay and the growth inhibition assay (or MTT assay). Both methods are widely used, although protocols may differ from one laboratory to another.

The clonogenic assay (Puck, 1955) measures the ability of cells to proliferate and form colonies following exposure to the drug under test and has been used extensively in the assessment of tumour cell lines (Hill, 1983) and freshly biopsied tumour tissue (Hamburger, 1977 and Courtenay, 1978). The cytotoxic potential of anti-cancer drugs is therefore measured as an anti-proliferative effect. Cytotoxicity is measured as a percentage of the number of colonies formed in comparison to untreated cells.

The MTT growth inhibition assay measures the respiration of cells as a function of the ability of mitochondria to reduce a tetrazolium-based compound (3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; MTT) to a blue formazan product (Mosmann, 1983 and Alley, 1988). This product absorbs light at a wavelength of 570nm and can be quantitated using a spectrophotometer, in the form of a 96 well plate-reader. Cytotoxicity is measured against untreated control cells. This method (Plumb, 1989) is an adaptation of the tetrazolium dye-based microtitration assay described previously (Carmichael, 1987).

For both the clonogenic and MTT assays, cytotoxicity is quantified in the form of an IC_{50} value; the concentration of drug, which results in a 50% cell kill. This represents a 50% reduction in colony number compared to control untreated cells for the clonogenic assay and a 50% reduction in optical density at 570nm compared to that of cells in the control untreated wells for the MTT assay. Finally, following analysis of

these factors, possible correlations between levels of enzyme expression and 5-FU cytotoxicity could be investigated.

2.2 Materials and Methods

2.2.1 Cell Lines

2.2.1.1 Source and characteristics

Analysis of the proteins regulating 5-FU cytotoxicity was performed on 5 human colon adenocarcinoma cell lines.

CELL LINE	CHARACTERISTICS	SOURCE	REFERENCE
DLD-1	Human Colon Adenocarcinoma	ATCC, CCL 221	Dexter, 1979
BE	Human Colon Adenocarcinoma	Dr Jane Plumb Department of Medical Oncology, University of Glasgow	Seigel, 1990
HT-29	Human Colon Adenocarcinoma	ATCC, HTB 38	Fogh, 1975
CACO-2	Human Colon Adenocarcinoma	ATCC, HTB 37	Fogh, 1970
LOVO	Human Colon Adenocarcinoma	ATCC, CCL 229	Drewinko, 1976

Table 2.1 Source and characteristics of the five colon cell lines used in this study.

2.2.1.2 Chemicals and reagents

DMEM, sodium bicarbonate, L-glutamine, foetal calf serum, penicillin and streptomycin and Trypsin were obtained from Gibco BRL (Paisley, UK). Ham's F10 medium was from SIGMA (Poole, UK), and diaminoethanetetra-acetic acid, disodium salt (EDTA) was obtained from Fisons Scientific Equipment (Loughborough, UK).

2.2.1.3 Routine cell maintenance

All cell lines were maintained in a combined Ham's F10 and DMEM medium supplemented by sodium bicarbonate (0.075%), L-glutamine (2mM), foetal calf serum

(10%, v/v), penicillin (50 U/mL) and streptomycin (50mg/mL). Cells were sub-cultured at weekly intervals at a 1:50 dilution and medium was replenished every 48 hours. All cell lines were incubated at 37°C in an atmosphere of 2% CO_2 in air.

Cells were sub-cultured by removal of all medium from the 75cm^2 flask and subsequent addition of 2.5 mLs of phosphate buffered saline (PBS) containing EDTA (1mM) and trypsin (0.25%) to the flask. Cells were incubated with the trypsin solution for 5-10 minutes, depending on the cell line, to allow dissociation of the cells in the monolayer. Trypsin was inactivated when the cells were resuspended in 7.5 mLs of 10% serum-containing medium. This suspension was diluted to the appropriate seeding concentration in new sterile tissue culture flasks.

2.2.1.4 Mycoplasma testing

All cell lines were free of mycoplasma as confirmed by monthly screening. Cells were fixed with ice-cold glacial acetic acid (25%, v/v) in methanol and stained with the fluorescent DNA stain Hoescht 3328 (SIGMA Chemical Company, Poole, UK) at a concentration of 100 ng/mL for 15 minutes at room temperature (Chen, 1977). Plates were then examined under a fluorescent microscope (Polyvar Microscope, Reichert, Leica Ltd, Milton Keynes, UK) for visible evidence of infection, i.e. characterised by extra-nuclear staining.

2.2.2 Growth kinetics

2.2.2.1 Chemicals and reagents

See section 2.2.1.2

2.2.2.2 Estimation of cell doubling times

The doubling time of each cell line was measured by counting the number of cells in the wells of a 24-well plate (Corning). Adherent cells were detached from

routine culture flasks using 0.05% trypsin-EDTA as described in section 2.2.1.3. Micro-titre plates (24-well) were seeded at a density of $2x10^4$cells/mL, with 1mL per well. After allowing cell attachment and growth to proceed for 24 hours in a humidified atmosphere (2% CO_2), cells in three wells were counted using a Coulter counter. Medium was replenished in the remaining wells daily to maintain optimal growth conditions. Sampling was continued at daily intervals for 11 days.

The pattern of cell growth was visualised by plotting log cell number against time. The population doubling time was then measured as the rate of growth in the linear section of the growth curve, corresponding to the exponential growth phase.

2.2.3 Polyacrylamide Gel Electrophoresis and Western Blot Analysis

This assay involves the solubilisation of cells using sodium dodecyl sulphate (SDS), followed by electrophoresis through a polyacrylamide gel, where proteins are separated on the basis of molecular size, a method developed by Laemmli (1970). The resolved proteins on the gel are transferred (by electroblotting) to a nitrocellulose membrane. Immobilised proteins are then visualised by reaction with antibodies specific to the protein of interest. The method of detection of these complexes is enhanced chemiluminescence (ECL). ECL is achieved by performing the oxidation of luminol by the Horse-radish Peroxidase (HRP) in the presence of chemical enhancers such as phenols (Whitehead, 1979). Immediately following oxidation, the luminol is in an excited state which then decays to ground state via a light emitting pathway. This can be detected by a short exposure to light sensitive autoradiography film.

2.2.3.1 Chemicals and Reagents

Triton X-100, sodium deoxycholate, phenylmethylsulfonylfluoride (PMSF), sodium orthovanadate, β-mercaptoethanol, bovine albumin, and Tween 20 (Polythylene-sorbitan mono laurate) were purchased from SIGMA (Poole, UK). Sodium chloride

(NaCl), sodium dodecyl sulphate (SDS), dithiothreitol were obtained from BDH Laboratory Supplies (Poole, UK). Bio-Rad protein dye was purchased from Bio-Rad Laboratories GmbH (Hertfordshire, UK) and the Perkin-Elmer Lambda 2 UV/Vis spectrometer was purchased from Perkin Elmer (Buckinghamshire, UK). Tris was obtained from GIBCO (Paisley, UK) and hydrochloric acid (HCl), glycerol, glycine and diaminoethanetetra-acetic acid, disodium salt (EDTA) were purchased from Fisons (Loughborough, UK). Immobilon-P nitrocellulose transfer membrane and a Milliblot™ graphite electroblotter were purchased from Millipore (Watford, UK) and Marvel™ was obtained from Premiere Beverages (Stafford, UK). The Enhanced Chemiluminescence (ECL) kit was obtained from Amersham (Buckinghamshire, UK). XR medical film was obtained from Fuji (Tokyo, Japan) and the KODAK X-OMAT 480 RA Processor was obtained from KODAK Ltd. (Hemel Hempstead, UK) and the Molecular Dynamics Laser Densitometer was purchased from Protein Databases Inc. (New York, USA). P-GF.44c, a mouse anti-TP monoclonal antibody raised against human TP was supplied by Dr Roy Bicknell, Molecular Angiogenesis Group, ICRF Clinical Oncology Unit, University of Oxford. The TS106 mouse anti-TS antibody raised against human TS was supplied by Prof. Patrick Johnston, Queens University of Belfast, Northern Ireland. An anti-mouse secondary antibody was obtained from Amersham (Buckinghamshire, UK).

2.2.3.2 Cell Lysate Preparation

Cells were dissociated from routine culture flasks using 0.05% trypsin-EDTA, as described in section 2.2.1.3. Cells were seeded in 25cm^2 culture flasks at a density of 2x10^4 cells/mL in a volume of 5mLs of 10% serum containing medium. The cells were allowed to attach and grow for approximately 48 hours, until 70% confluence was reached. Cells were rinsed three times with ice-cold phosphate-buffered saline (PBS) and lysed by incubation with a buffer containing tris (25mM), NaCl (150mM), pH 7.5,

Triton X-100 (1%), sodium deoxycholate (1%), SDS (0.1%), dithiothreitol (1mM), sodium orthovanadate (100mM) and PMSF (10mM), for 20 minutes on a rocking table at 4°C. Cells were then removed from the tissue culture flasks using a cell scraper. Following centrifugation at 10,000g for 20 minutes in a refrigerated microcentrifuge, cytoplasmic protein concentration was quantified using the Bio-Rad protein assay (section 2.3.2.3) and aliquots stored at -70°C until required.

2.2.3.3 Measurement of protein content

The Bio-Rad protein assay was used to determine protein content in each cell lysate preparation. This assay is a colourimetric assay for the measurement of total protein concentration. The principle of the assay being that Coomassie brilliant blue G-250 dye will undergo a concentration dependant colour change in response to different concentrations of protein (by binding to basic and aromatic amino acid residues).

A series of known concentrations of bovine albumin standards were prepared (0-25μg/mL) in 800μL of water and 200μL of Bio-Rad dye. These were transferred to 1.5mL cuvettes and the optical density was measured at 595nm on a spectrophotometer. Test samples (supernatants from the crude cell lysate preparations), were diluted as appropriate and 10μL of each sample was added to 790μL of water and 200μL of Bio-Rad dye. A plot of protein concentration versus optical density at 595nm defined the standard curve from which the protein concentration of the unknown samples could be determined.

2.2.3.4 Electrophoresis

One hundred micrograms of crude cell lysate was size-fractionated by SDS-PAGE in a 6.5% acrylamide (30% w/v acrylamide, Bis Acrylamide Ratio 19:1) gel containing tris base (300mM), SDS (3mM), pH 8.9 with HCl. The stacking gel contained 4% acrylamide in tris (170mM) and SDS (5mM) pH 6.7 with HCl. Samples

were diluted 1:2 (v/v) in loading buffer; SDS (4%), tris (250mM), EDTA (0.05M), glycerol (20%), β-mercaptoethanol (5%) and bromophenol blue. Samples were then boiled for 3 minutes prior to electrophoresis. Electrophoresis of proteins was carried out at 60mA, 300V for 1 hour, followed by 5-10mA, 300V overnight, in electrode buffer; tris (100mM), glycine (100mM) and SDS (3.5mM).

2.2.3.5 Immunoblotting

A semi-dry graphite electroblotter was used to transfer the proteins from the gel onto a nitrocellulose membrane in a buffer consisting of tris (48mM), glycine (39mM) and SDS (0.037%). In order to block non-specific binding of the antibodies, the membranes were incubated in 5% Marvel™ (condensed milk) for 1 hour at room temperature. Membranes were washed in freshly prepared tris buffered saline containing tris base (10mM) and NaCl (150mM) pH 7.4 and 0.025-0.05% Tween 20 (TBST). Following washing, the membrane was incubated with the primary antibody diluted appropriately in blocking solution, for the appropriate time period. For example, TP antibody (P-GF.44c) was diluted 1:1000 (v/v) in blocking solution and incubated overnight at 4°C whereas the TS antibody (TS106) was diluted 1:500 in blocking solution and incubated for 1 hour at room temperature. The membrane was then washed 3 times (5 minutes each) in TBST prior to incubation with the secondary antibody linked to horseradish-peroxidase. This antibody was diluted 1:5000 (v/v) in blocking solution and incubated for 1 hour at room temperature. Following a final wash with TBST, the antibody/protein complex was visualised using an Enhanced Chemiluminescence kit (ECL™) and exposed to X-ray film. The film was processed using a KODAK X-OMAT 480 RA Processor. The density of the bands representing the protein of interest were quantified using a Molecular Dynamics Laser Densitometer with Image Analysis Software.

2.2.4 Cytotoxicity Assays

2.2.4.1 Chemicals and reagents

5-FU and MTT were purchased from SIGMA (Poole, UK). Dimethylsulphoxide (DMSO), was obtained from Fisons Scientific Equipment (Loughborough, UK) and Gram's crystal violet solution was from BDH Laboratory Supplies (Poole, UK). 96 well microtitre plates and $25cm^2$ culture flasks were obtained from Bibby Sterilin (Stone, Staffordshire). A Precision Microplate Reader was purchased from Molecular Devices Corporation (West Sussex, U.K.). An Artex colony counter was purchased from Artex Systems Corporation.

2.2.4.2 Growth inhibition assay (MTT assay)

Cells were dissociated from routine culture flasks using 0.05% trypsin-EDTA as described in section 2.2.1.3. Cells were seeded in 96 well microtitre plates in a volume of 200μL per well, at a density of $2.5x10^3$cells/mL. Two hundred microlitres of complete medium was added to cell-free wells as controls. After allowing cell attachment and growth to proceed for 48 hours in a humidified atmosphere of 2% CO_2 in air, concentrations of 5-FU from $2.5x10^{-7}$ to $5x10^{-5}$M were added in a volume of 200μL. The drug was replenished daily when incubation times were greater than 24 hours. At the end of the drug treatment period the medium was replaced with drug-free medium and the cells were allowed to grow for a further 72 hours with daily replenishment of medium. After 72 hours the cells were assayed for growth inhibition. MTT at a concentration of 0.5mg/mL was added to the medium in the wells and the plates were incubated in the dark at 37°C in the same humidified atmosphere. After 4 hours the MTT/medium was removed from the wells and the MTT-formazan crystals were dissolved in 200μL of DMSO, followed by 25μL of glycine buffer (0.1M glycine, 0.1M NaCl, pH 10.5). The optical density of the blue formazan product was measured

on a Precision Microplate Reader at a wavelength of 570nm and dose-response curves and IC_{50}'s were calculated using the SOFTmax 2.32 programme. IC_{50}'s were calculated as the concentration of drug required to inhibit cell growth by 50% compared to the controls, visualised as a 50% reduction in optical density at 570nm. A typical dose-response curve consisted of 8 drug concentrations with 8 wells per concentration and 3 plates were analysed within each experiment.

2.2.4.3 Clonogenic assay

Cells were seeded in $25cm^2$ tissue culture flasks (Corning) in 5mLs of medium, at a density of $7.75x10^4$cells/mL. After allowing cell attachment and growth to proceed for 48 hours, concentrations of 5-FU were added from $2.5x10^{-7}$ to $5x10^{-5}$M in 5mLs of medium. A control flask with drug-free medium was also included. The drug was replenished daily where incubation times were greater than 24 hours. At the end of this period, the cells in the control flask were removed by treatment with trypsin (0.05%) and counted. A total of 10^3 cells were subsequently seeded into four 60x15mm petri-dishes in a volume of 5mLs. The drug-treated flasks were processed in the same manner as the controls and colonies were allowed to grow for an additional 10 days under conditions of temperature and CO_2 previously described, at which point they were fixed with methanol and stained with 0.1% crystal violet. Following counting of the colonies on an Artex colony counter, a graph was plotted of drug concentration against colony number represented as a percentage of the control number. IC_{50}'s were calculated as the concentration of drug, which resulted in a 50% reduction in colony number compared with the controls.

2.3 Results

2.3.1 Growth Kinetics

The estimated population doubling times of the 5 cell lines, taken from a single experiment, are outlined in Table 2.2 and growth curves are shown in Figure 2.1. A 1.7-fold range in population doubling times was observed among the 5 cell lines.

Cell line	Estimated Population Doubling Time (hrs)
BE	36.0
HT-29	38.4
LOVO	28.8
DLD-1	40.8
CACO-2	50.4

Table 2.2 Estimated population doubling times of the 5 colon adenocarcinoma cell lines. Cells were counted daily for 11 days as described in section 2.2.2.2.

2.3.2 TP protein expression

TP enzyme was detected in only LOVO cells (lane 6) with a band corresponding to a protein of 55kDa in size, consistent with the band for human recombinant TP in lanes 1-3, Figure 2.2. TP protein was not detected in CACO-2, DLD-1, LOVO and HT-29 cells.

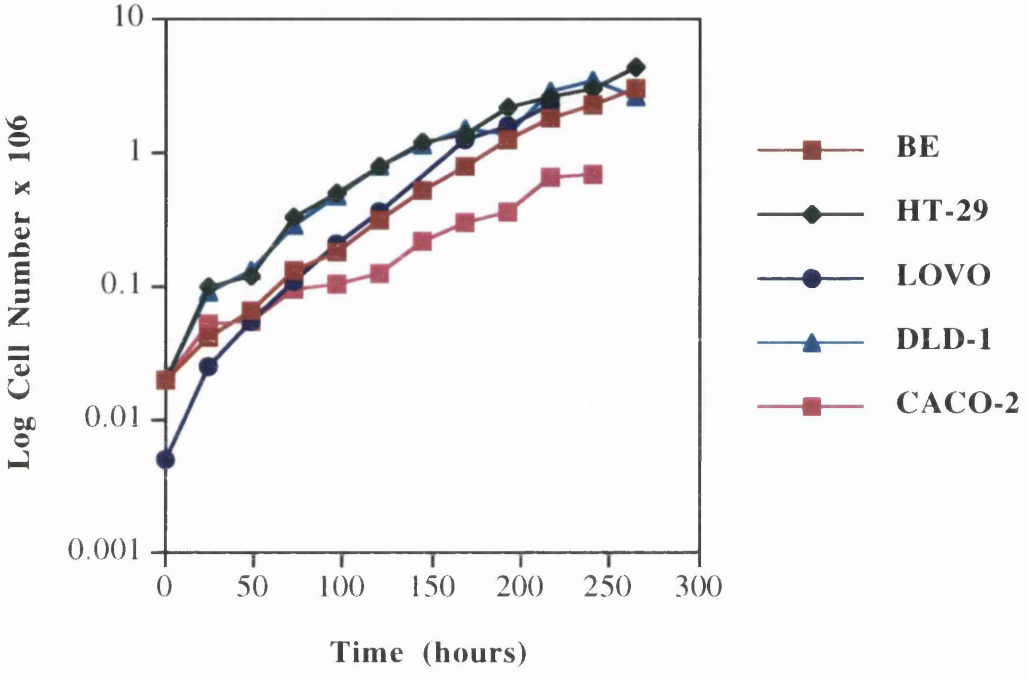


Figure 2.1 Cell growth curves for colon adenocarcinoma cell lines; BE, HT-29, LOVO, DLD-1 and CACO-2. Values are means and standard deviations of 3 counts per day.

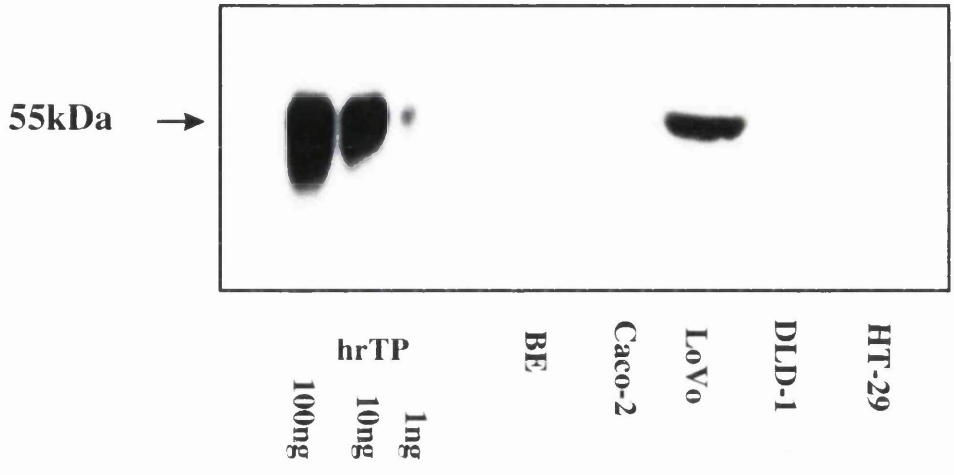


Figure 2.2 Western immunoblot of human recombinant TP (100, 10 and 1ng), BE, CACO-2, LOVO, DLD-1 and HT-29 cells with PGF.44c anti-TP antibody. For analysis of TP protein levels, cells were lysed and cytosols prepared by centrifugation at 10,000g. 100µg of cytosol was size-fractionated by SDS-polyacrylamide gel electrophoresis. Proteins were electroblotted onto nitrocellulose membranes and incubated with PGF.44c, a mouse monoclonal antibody raised against human TP, followed by an anti-mouse secondary antibody linked to HRP. Protein-antibody complexes were visualised using enhanced chemiluminescence.

2.3.3 TS protein expression

TS protein was detected in all five cell lines with varying degrees of expression, Figure 2.3. TS protein (as a positive control) is not available commercially, however the bands corresponded to a protein approximately 36kDa in size; the molecular weight of TS (Johnston, 1991). A 13.1-fold range in TS protein expression was observed, as outlined in Table 2.3. There was no correlation between TS expression and population doubling time(r=0.4, Figure 2.4).

Cell Line	Relative TS expression
HT-29	4.7
CACO-2	1
DLD-1	9.0
LOVO	5.3
BE	13.1

Table 2.3 TS protein expression in colon cell lines relative to CACO-2 from a single Western immunoblot experiment. TS protein expression was evaluated by immunoblotting with TS106 antibody. Antibody/protein complex was visualised using enhanced chemiluminescence as illustrated in Figure 2.3. These results are based on a single experiment.

2.3.4 5-FU Cytotoxicity

5-FU IC_{50} data (for 24 hour drug exposure) obtained using both the clonogenic and the MTT assays are outlined in Table 2.4. Representative dose-response curves are shown in Figure 2.5, with (A) and (B) portraying the clonogenic and MTT assays respectively.

From Table 2.4 it can be seen that there is a 3.3-fold range in sensitivity of the colon cells to 5-FU as measured by the MTT assay and a 4.1-fold range for the clonogenic assay. All IC_{50} values are in the lower micromolar range (8-36μM). To confirm whether the observed range of 5-FU sensitivities measured by both assays correlated, the two sets of data were compared (Figure 2.6). Chemosensitivity measured by the two assays was strongly correlative (r=0.89).

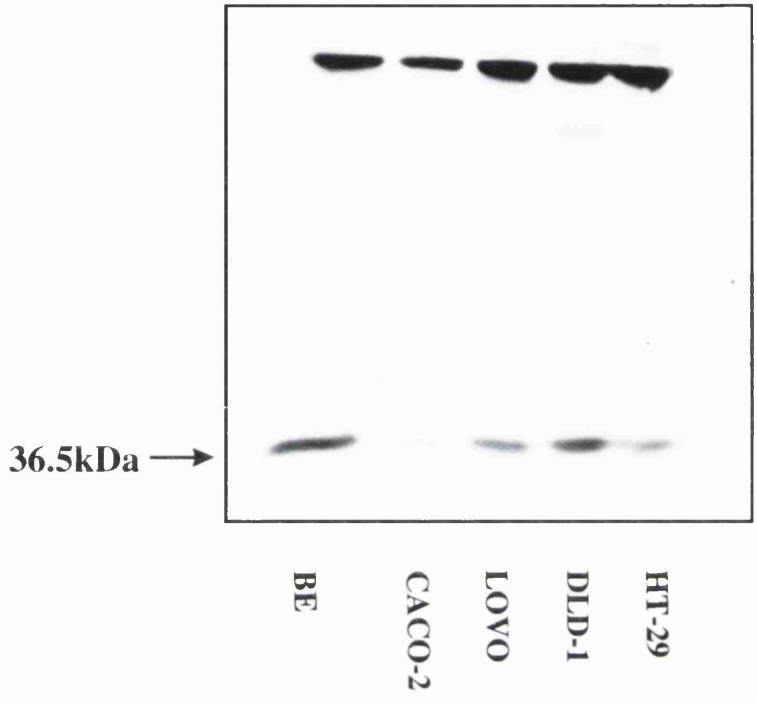


Figure 2.3 Western immunoblot of BE, CACO-2, LOVO, DLD-1 and HT-29 cells (lanes 1-5 respectively) with TS106 anti-TS antibody. For analysis of TS protein, cells were lysed and cytosols prepared by centrifugation at 10,000g. 100µg of cytosol was size-fractionated by SDS-polyacrylamide gel electrophoresis. Proteins were electroblotted onto nitrocellulose membranes and incubated with TS106, a mouse monoclonal antibody raised against human TS, followed by an anti-mouse secondary antibody linked to HRP. Protein-antibody complexes were visualised using enhanced chemiluminescence.

Likewise when ranked in order of sensitivity (most sensitive to least sensitive), the same ranking was shown for both assays, except for HT-29 and CACO-2 where IC_{50}'s were very similar (Table 2.4).

To determine whether TS enzyme expression correlates with 5-FU sensitivity, relative protein expression (Table 2.2) was compared with 5-FU IC_{50}s obtained using the MTT assay (Table 2.4). The level of TS protein expression was found to correlate with 5-FU sensitivity (r=0.87), Figure 2.7.

CELL LINE	IC_{50}(μM) (Rank)	
	Clonogenic Assay	**Growth Inhibition (MTT) Assay**
DLD-1	**23** (4)	**30.3±12.1** (4)
BE	**36** (5)	**30.4±3.7** (5)
HT-29	**12.5** (2)	**9.2±3.4** (1)
CACO-2	**8.8** (1)	**11.2±3.2** (2)
LOVO	**17** (3)	**20.3±8.1** (3)

Table 2.4 5-FU IC_{50} measurements in colon cell lines, measured by the growth inhibition (MTT) assay and the clonogenic assay. Representative 5-FU IC_{50} values obtained from dose-response curves pictured in Figure 2.5. MTT IC_{50} values are means and standard errors of three 96-well plates, 8 wells per plate.

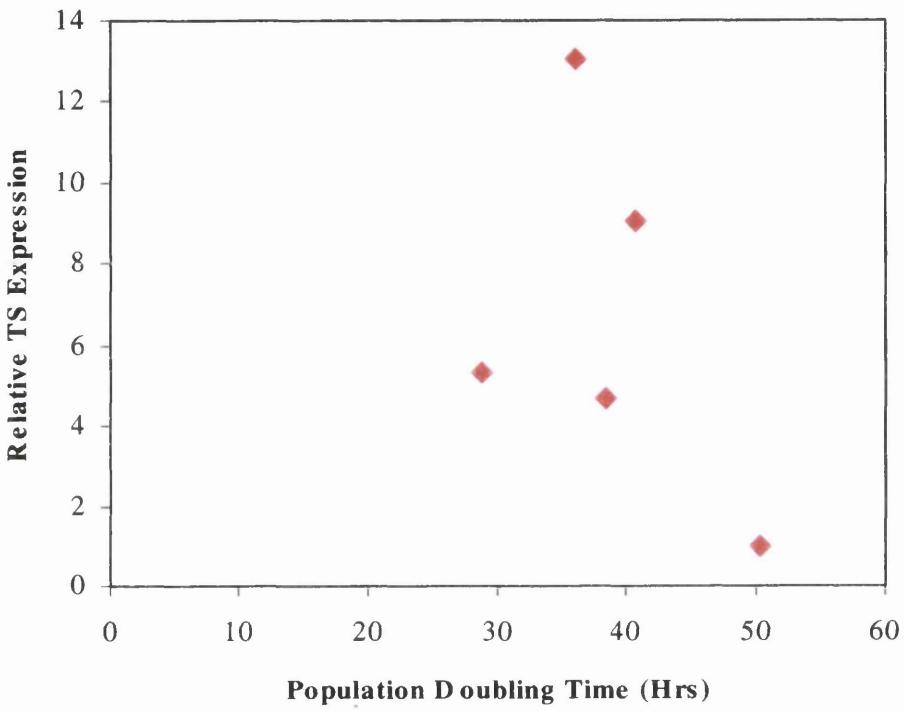


Figure 2.4 TS protein expression versus population doubling time. Linear regression analysis (y=mx+c); TS expression = -0.3 (Doubling time) + 16.9, r=0.4.

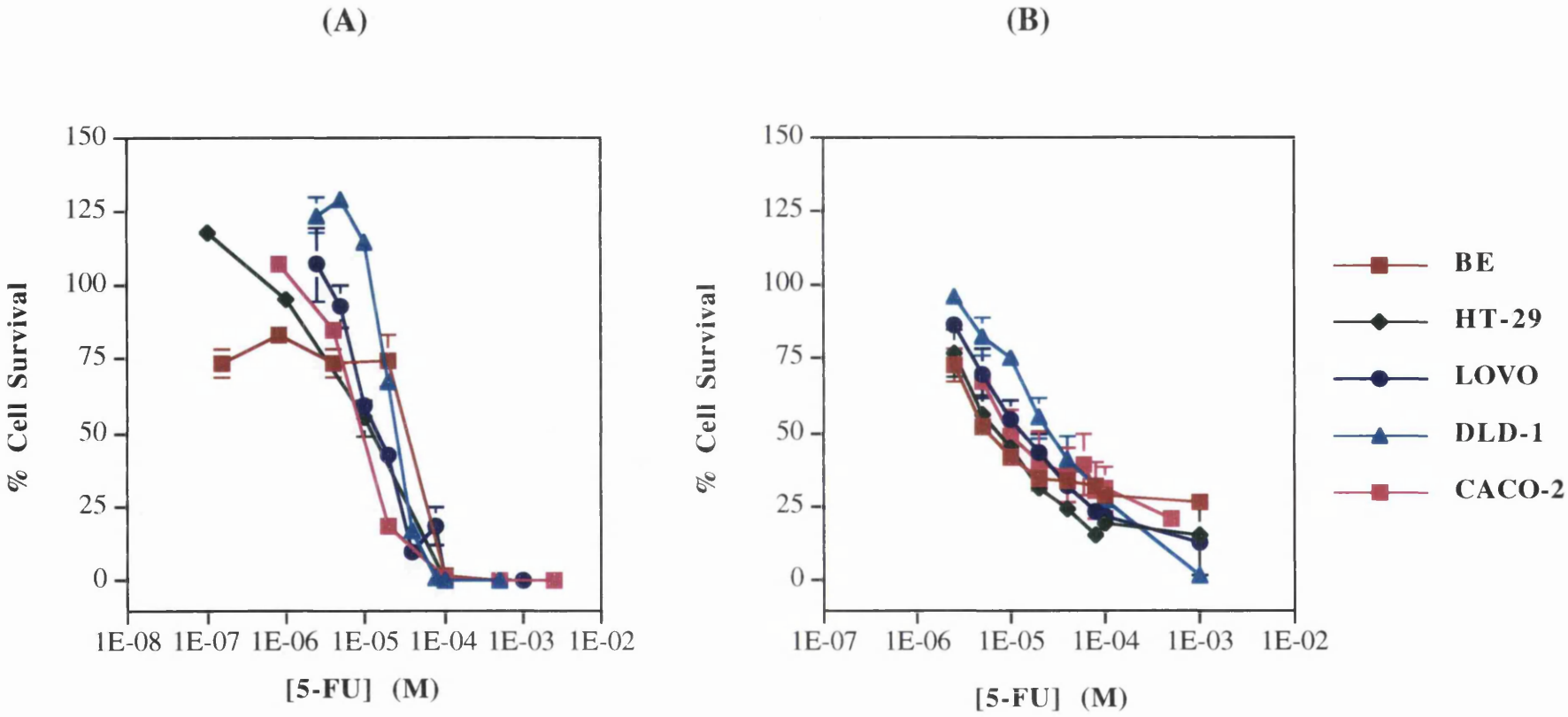


Figure 2.5 5-FU cytotoxicity in colon adenocarcinoma cell lines; BE, HT-29, LOVO, DLD-1 and CACO-2. Two methods of measuring cytotoxicity were employed; **The clonogenic assay (A)**, where cells were treated with increasing concentrations of 5-FU in 25cm^2 flasks for 24 hours, then seeded in four 60x15mm petri-dishes and allowed to grow for 10 days before colonies were counted. IC_{50}' s were defined as the concentration of drug required to cause a 50% reduction in colony number when compared to untreated controls. Alternatively, **the growth inhibition assay/MTT assay was employed, (B)**. Cells were treated with increasing concentrations of 5-FU in 96-well plates for 24 hours. Growth inhibition was measured after 3 days growth in drug-free medium, by incubation with MTT(50µg/mL) for 4 hours. IC_{50}'s defined as the concentration of required to cause a 50% reduction in optical density at 570nm compared to drug-free controls. Values are means and standard errors of 3 plates, with 8 wells per drug concentration.

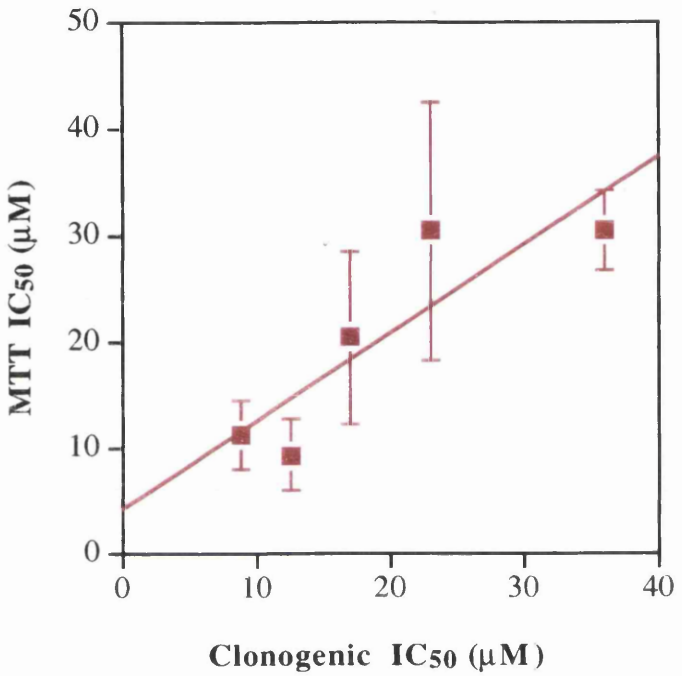


Figure 2.6 MTT Assay versus Clonogenic Assay. Plot of 5-FU IC_{50} as measured by the MTT assay versus the Clonogenic Assay. Linear Regression Analysis (y=mx+c); MTT IC_{50} = 0.83 (Clonogenic IC_{50}) + 4.11, r=0.88.

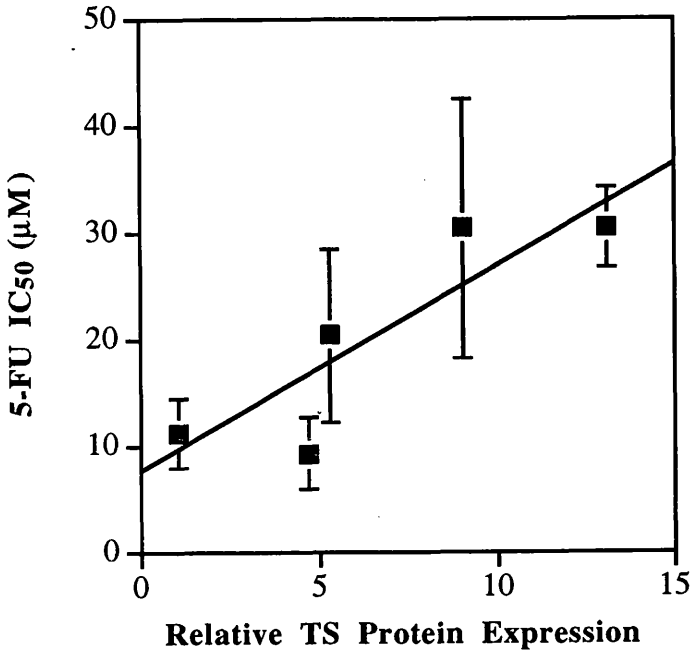


Figure 2.7 TS expression versus 5-FU IC_{50}. Plot of 5-FU IC_{50} (from MTT Assay, Table 2.4) versus TS protein expression (from Western blot analysis, Figure 2.3, Table 2.3) in colon adenocarcinoma cell lines BE, HT-29, LOVO, DLD-1 and CACO-2. Linear regression analysis (y=mx + c); 5-FU IC_{50} = 1.92 (TS expression) + 7.56, r=0.87.

2.4 Discussion

Five colon adenocarcinoma cell lines have been characterised for their growth patterns and the expression of two important enzymes responsible, in part, for the cytotoxicity of the pyrimidine analogue 5-FU. This study was carried out to consider the importance of these characteristics in relation to the intrinsic sensitivity of colon cancer cell lines to 5-FU.

2.4.1 Population Doubling Times

There was only a 1.7-fold range in population doubling times (29-50hrs) within the five cell lines. LOVO cells were the fastest growing and also the only cell line in which TP protein expression could be detected by Western immunoblotting. In addition, a 30 minute exposure of the x-ray film did result in a faint band at approximately 55kDa in HT-29 cells.

2.4.2 TP

No correlations could be made with respect to TP expression and other characteristics such as 5-FU cytotoxicity, due to the lack of detection of TP by Western immunoblotting (later in this study a more sensitive method for measuring TP activity was developed, see chapter 5).

Interestingly, TP protein was detected in DLD-1 cell lines earlier in the study, however it was discovered at a later mycoplasma test that the cells were mycoplasma positive. The presence of TP protein could be attributable to the mycoplasma infection itself since it is known to have high TP activity (Levine, 1972).

A narrow range in sensitivities to 5-FU was observed. Cytotoxicity measured by the MTT assay was, in general, in concordance with that measurable by the clonogenic assay method. There was no apparent relationship between 5-FU IC_{50} and TP protein

expression measured by Western immunoblotting. LOVO cells, despite being the only cell line with detectable levels of TP protein, were not the most sensitive to 5-FU. 5-FU has multiple routes of anabolism to cytotoxic nucleotides. TP may not, therefore, be the rate-limiting step for the intrinsic sensitivity to 5-FU in this particular cell line. In cell lines such as CACO-2, where TP was not detected yet 5-FU sensitivity was relatively high, other enzymes or anabolic pathways must predominate.

TS protein was detected in all five cell lines, with a 5.9-fold range in expression. LOVO cells, which were the fastest growing and the only cells to have detectable levels of TP protein, had a high expression of TS protein in relation to the other cell lines. There was no correlation between TS expression and population doubling time (r=0.4).

There was a correlation (r=0.87) between TS protein expression and sensitivity to 5-FU. Other studies have demonstrated a similar relationship between TS protein levels and response to 5-FU in cell lines (Washtein 1984 and Johnston, 1992), experimental tumours (Spears, 1982) and in the clinical setting (Swain, 1989). Johnston *et al* demonstrated that the levels of TS enzyme measured using the same TS antibody (TS 106), were higher in 5-FU insensitive cell lines.

These data have been confirmed clinically where response to 5-FU-based therapy significantly correlates with TS protein levels and mRNA in the primary tumour from colorectal and gastric cancer patients (Johnston, 1995 and Lenz, 1993). Those colorectal and gastric patients who responded to treatment had a low mean TS protein level whereas those who did not respond had high TS expression. More recently, studies with colon and breast tumour biopsy specimens demonstrated that TS-positive colon specimens, from previously untreated patients showed a 30% reduction in response to 5-FU.

In the following chapter TP expression was measured in human tissue to determine whether the cell line data reflects the clinical situation. In addition, it may be that TP

activity is more relevant than protein expression, therefore a method was developed to measure enzyme activity in cell lines and human tissues.

CHAPTER 3

3. TP Protein Expression in Human Colon Tumour and Normal Tissue

3.1 Introduction

Thymidine phosphorylase (TP) has diverse roles within cells. These include maintaining steady-state levels of thymidine, which is required for DNA synthesis and mediating physiological and pathological angiogenesis. TP also catalyses the conversion of 5-FU to the active metabolite 5-FdUrd which in turn is converted to 5-FdUMP resulting in inhibition of the enzyme TS.

TP (originally known as PD-ECGF) was first isolated from platelets (Miyazono, 1987) and has since been demonstrated in placenta (Usuki 1990), spleen, peripheral lymphocytes, lymph nodes and macrophages in liver and lung tissues (Yoshimura, 1990).

TP has a role in cancer cell proliferation with increased expression found in many tumours compared to adjacent normal tissues including carcinomas of the colon, stomach and ovaries (Yoshimura, 1990). Elevation of TP has been demonstrated by both the ribonuclease protection assay (RPA) and immunohistochemistry in breast tumours (Fox, 1996). Increased TP protein expression is found in invasive ductal breast carcinoma tissue compared to normal ducts of the same carcinoma tissue and benign lesions including fibrocystic disease and fibroadenomas (Toi, 1995). In patients with gastric carcinomas who have received no chemotherapy or radiation therapy prior to surgery, TP is detectable in tumours but not in normal gastric mucosa (Maeda, 1996).

Studies measuring TP mRNA expression found a similar trend as that seen with protein expression. In ovarian cancer there is a significant elevation in TP mRNA in malignant tumours compared to benign tumours (Reynolds, 1994). In 45 primary

bladder tumours and eight normal bladders evaluated by O'Brien and colleagues (1995), the invasive tumours had 33-fold greater TP mRNA than superficial tumours and 260-fold greater TP mRNA than normal bladder. TP enzymatic activity has also been measured in tumours and this will be discussed in more detail in chapter 5. To summarise some of these studies however, TP activity is higher in gastric, colorectal, cervical, breast and liver tumours than normal tissues (Luccioni, 1994; Miwa, 1986; Zimmerman and Seidenberg, 1964).

In the current chapter, human colon tumour and normal tissues were evaluated for TP protein expression by Western immunoblotting with the P-GF.44c monoclonal antibody raised against human TP protein used in chapter 2. This allowed comparison of human tumours with cell lines since TP protein expression was previously evaluated in 5 human colon adenocarcinoma cell lines (section 2.3.2) and was detected in only one of them (LOVO). In chapter 5 TP activity is measured in human tumour cell lines and a different panel of 10 human colon tumour and normal tissues.

3.2 Materials and Method

3.2.1 Chemicals and reagents

See Section 2.2.3.1

3.2.2 Preparation of tissue samples

Nine human colon tumour/normal biopsy pairs were obtained from Dr. Richard Lamb (Beatson Institute for Cancer Research, Glasgow). Patients had received no chemotherapy or radiation therapy prior to surgery. Information such as grade, size and malignancy was not available and therefore a correlation between TP expression and these factors could not be assessed.

Tissue samples were weighed and homogenised in 5w/v of lysis buffer (see section 2.2.3.2). Homogenates were centrifuged at 10,000g at 4°C for 20 minutes and

supernatants were stored at -70°C until required. The protein concentration of the supernatant was measured using the Bio-Rad assay method outlined in section 2.2.3.3.

3.2.2.1 Western blotting

Western Blot analysis was carried out using the P-GF.44c antibody as described in section 2.2.3.4-2.2.3.5. The results were quantified by densitometric scanning.

3.3 Results

TP protein was detected in all tumour and normal colon biopsy tissues analysed. A representative Western immunoblot is shown in Figure 3.1. Densitometry of the Western immunoblots for all colon pairs is illustrated in Figure 3.2. TP protein is consistently higher in tumours when compared with patient matched normal colon mucosa (1.2- to 8.7-fold). Heterogeneity of TP protein expression was demonstrated with a 14.5-fold range in expression between normal colon mucosas and a 4.7-fold range in expression in between the tumours.

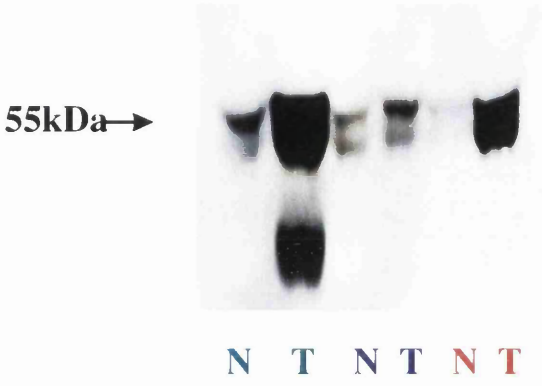


Figure 3.1 Representative western immunoblot of three human colon normal/tumour (N/T) biopsy pairs with PGF.44c anti-TP antibody. For analysis of TP protein levels, biopsies were homogenised and cytosols were prepared by centrifugation at 100,000g. For analysis of TP protein levels, cells were lysed and cytosols prepared by centrifugation at 10,000g. 100µg of cytosol was size-fractionated by SDS-polyacrylamide gel electrophoresis. Proteins were electroblotted onto nitrocellulose membranes and incubated with PGF.44c, a mouse monoclonal antibody raised against human TP, followed by an anti-mouse secondary antibody linked to HRP. Protein-antibody complexes were visualised using enhanced chemiluminescence.

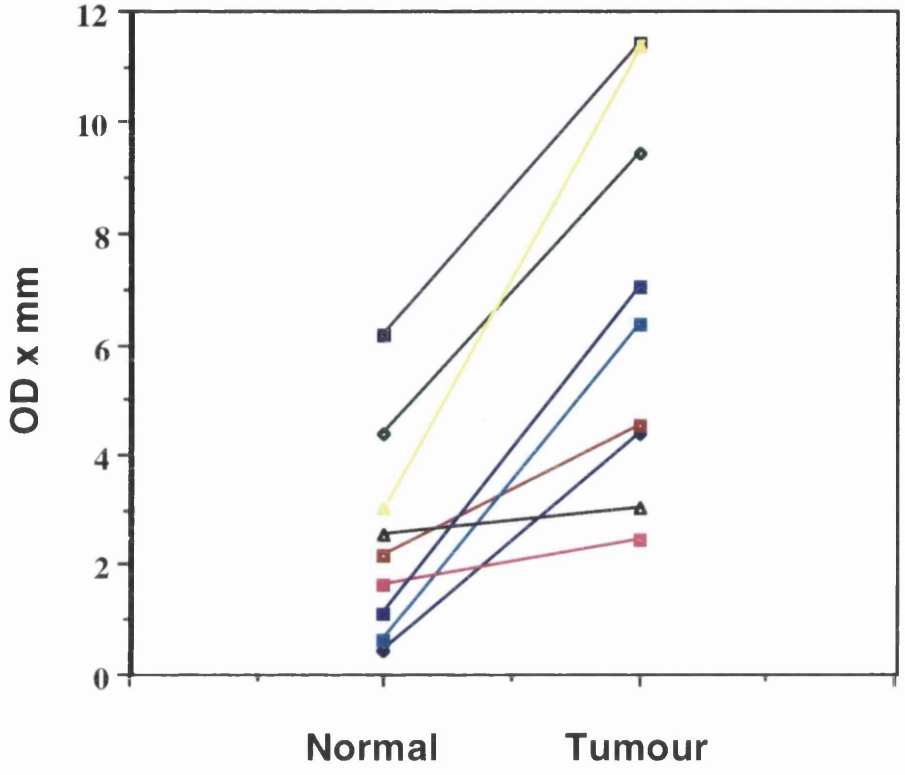


Figure 3.2 TP Expression in tumour/normal biopsy pairs. Plot of relative TP protein expression in colon normal and tumour biopsy pairs.

3.4 Discussion

TP expression is elevated in many tumour types compared to adjacent normal tissue. TP protein expression was measured in nine human colon tumour/normal mucosa biopsy pairs and was higher in all tumours tissues compared to their normal tissue counterparts. The current study is consistent with current literature on TP mRNA and activity.

Studies have correlated TP expression in tumours with other factors such as vascularity, response to chemotherapy and overall survival. In a study of 100 invasive ductal breast carcinoma tissues, TP expression correlated with microvessel density, a marker for angiogenesis (Toi, 1995). Fox *et al* also found that strong TP staining was prominent at the tumour periphery where angiogenesis is most active (Fox, 1996). The ovary is one of the few sites where physiological angiogenesis occurs and an increase in TP mRNA was demonstrated in sections of malignant ovarian tumours compared with benign ovarian tumours (Reynolds, 1994). Microvessel density was also significantly higher in TP positive tumours compared with TP negative gastric tumours (Maeda, 1996). These data emphasise the involvement of TP in the process of angiogenesis which in turn has prognostic significance. Patients with TP positive gastric tumours are more likely to develop hepatic metastasis (Maeda, 1996) and have a shorter survival than those with TP negative tumours. Likewise, a worse prognosis was also described for patients with TP positive lung tumours (Koukourakis, 1997).

A study of TP expression in 91 curative resected primary colorectal carcinomas demonstrated that tumours where more than 5% of carcinoma cells stained positive with TP antibody, had an increased depth of invasion, increased number of metastatic lymph nodes, greater vessel invasion, more advanced Duke's stage and higher incidence of recurrence. Patients with Duke's C stage tumours which were TP-positive, demonstrated

shorter survival time, compared with TP-negative tumours of the same stage ($P<0.05$). It would appear therefore that up-regulation of TP defines a more aggressive tumour phenotype.

A study of 240 primary breast carcinomas examined the relationship between TP protein and mRNA, and correlations between TP protein and other variables such as tumour grade, size and prognosis (Fox, 1996). In those women with node positive disease and TP-positive tumours, relapse free survival was better than in other patient groups thus contradicting the results obtained for lung cancer. This difference may be related to the different agents used in their treatment. Breast cancer is usually treated with fluoropyrimidine-based therapy and TP is an important activating enzyme. Lung cancer is rarely treated with fluoropyrimidines therefore increased TP may not provide any therapeutic benefit. A pilot study of 328 invasive breast carcinomas has indeed shown that there is a correlation between TP levels and response to treatment with Cyclophosphamide, Methotrexate and 5-Fluorouracil (CMF). This resulted in a significant survival advantage (relapse-free and overall survival) following treatment with CMF (Fox, 1997).

As can be seen in Figure 3.2, there is interpatient heterogeneity of expression of TP in the normal colon. In a recent immunohistochemical study, Fox and colleagues also demonstrated heterogeneous TP staining in different human tissues and also between adjacent cells in the same tissue (Fox, 1996). The same heterogeneity of TP expression was observed in tumour tissue in the current study. This is consistent with studies of other tumour types including breast, lung, ovarian, colorectal, gastric and bladder tumours (Toi, 1995; Fox, 1996; Heldin, 1993; Reynolds, 1994; Luccioni, 1994; Maeda, 1996; O'Brien, 1995). Intra-tumoural heterogeneity of TP expression may be explained by the fact that tumours include stroma, inflammatory infiltrate and inflammatory cells such as macrophages which have high TP expression (Fox, 1996).

Heterogeneity between tumours may be explained by the grade or vascularity of the tumour. In breast cancer TP expression inversely correlates with size and grade of the tumour suggesting that TP plays an important role in the initial stages of angiogenesis in these tumours (Fox, 1996). In malignant ovarian tumours TP is expressed at a significantly higher level compared with benign tumours (Reynolds, 1994).

These data differ from that obtained from human tumour cell lines (section 2.3.2, Figure 2.2) where TP was detected in only 1 out of 5 colon tumour cell lines. Luccioni and colleagues evaluated various pyrimidine nucleotide metabolising enzymes for their relative activity in normal tissues, primary tumours and xenografts. TP activity was lower in xenografts than in the primary tumours from which they were derived (Luccioni, 1994). TP activity was also greater in tumours and xenografts than in colon cell lines (including HT-29 and CACO-2), which is similar to the findings described in section 2.3.2. One possible explanation is that there is down-regulation of TP *in vitro.* This reduction in TP activity may be due to an adaptation of the tumour cells to alternative growth conditions *in vitro.* For example, there may be an increased requirement for thymidine due to an increased growth rate or lower availability of thymidine. Another explanation is that TP protein detected in tumours could be derived from cells other than neoplastic cells such as macrophages or endothelial. Alternatively, it may be that the principle role of TP in tumours is in the process of angiogenesis, a process no longer required nor driven in culture.

Colon cell lines therefore, may not be representative of colon tumours when evaluating 5-FU cytotoxicity since TP appears to be down-regulated when tumour cells are introduced into culture. For the purposes of this thesis however, comparisons will be made between cells in which TP activity/expression has been increased by DNA transfection. This provides a controlled environment in which to evaluate the impact of TP on 5-FU activity.

The current study has shown that colon cancer behaves in the same manner as gastric, ovarian and breast cancers in that the tumours express higher levels of TP compared with their normal tissue counterparts. Interpatient tumour TP expression is heterogeneous as is expression in normal colon mucosa. The current data on the prognostic impact of TP staining suggests a correlation between TP and response to CMF in breast cancer (Fox, 1997). It may be of therapeutic benefit in colon cancer therefore to select patients with low intra-tumoural levels of TP and use gene therapy to transiently increase the expression of TP. This may sensitise the tumour cells to the effects of fluoropyrimidines and transient gene expression may avoid the development of a more aggressive phenotype. This could be of significant benefit in colon cancer due to its inherent resistance to treatment. This hypothesis was explored in a colon tumour cell line and is described in the following chapters.

CHAPTER 4

4. Characterisation of colon cells transfected with TP cDNA

4.1 Introduction

The aim of this study was to transfect colon cells *in vitro* with the gene encoding TP, confirm it's expression and assess it's contribution to 5-FU cytotoxicity.

4.1.1 IFN-α and 5-FU

The mechanism by which the IFN-α exerts it's effect on 5-FU cytotoxicity *in vitro* has been evaluated. IFN-α treatment leads to increased cytotoxicity of 5-FU and some studies have demonstrated that this is at the level of TS (Elias, 1988, Schwartz, 1992, Chu, 1990 and Houghton, 1991). In a number of studies an increase in the activity of TP has been observed following exposure to IFN-α (Eda, 1993, Tevaearai, 1992 and Schwartz, 1992). The subsequent increase in 5-FU cytotoxicity was circumvented by exogenous thymidine, confirming that inhibition of thymidylate synthase occurred (Wadler, 1990; Elias, 1988 and Houghton, 1991).

Patient studies using this combination were initially promising (Wadler, 1989) however many groups have been unable to repeat Wadlers work (Pazdur, 1990; Kemeny, 1990; Huberman, 1991; Weh, 1992 and Martoni, 1995). There was a high incidence of side-effects particularly leucopenia, diarrhoea, mucositis and neurotoxicity. The most frequent side-effect was a flu-like syndrome induced by IFN.

The current study sought to evaluate whether an increase in the expression of TP alone, by DNA transfection, could enhance 5-FU cytotoxicity in colon cell lines *in vitro*. The cell line chosen for these studies was HT-29, a cell line in which the effects of IFN-α and 5-FU have previously been studied (Schwartz, 1992 and Tevaearai, 1992). HT-29 cells have also been evaluated earlier in the present study and were found to have no detectable levels of TP protein by Western blotting. The role of TP can therefore be

assessed in transfected cells compared with parental cells with no or very low TP activity. Colon cancer was chosen as the model for these studies since it is commonly treated with 5-FU and other fluoropyrimidines although response rates are disappointing.

If transfection of colon cells (HT-29) with TP cDNA confers increased sensitivity to 5-FU, this will provide direct evidence of the pharmacological interaction between 5-FU and IFN-α. More importantly, this pathway could also be exploited through the application of gene therapy techniques directing tissue-specific expression of TP in a transient fashion, rendering cells more sensitive to fluoropyrimidines such as 5-FU. As a consequence, the toxicity associated with the biochemical modulator IFN-α may be avoided.

4.1.2 DNA transfection

DNA transfection required first a decision regarding which method to use. There are four commonly used methods for the transfection of mammalian cells; calcium phosphate (Ca_2PO_4) precipitation, DEAE-Dextran-mediated gene transfer, electroporation and liposomal-mediated gene transfer.

Ca_2PO_4 precipitation is one of the most widely used techniques and involves the introduction of DNA to monolayer cultures via a precipitate which adheres to the cell surface. DNA is then taken up by the cells via endocytosis (Graham, 1973).

DEAE-Dextran works by a similar method to Ca_2PO_4 precipitation, in that it forms complexes with DNA which are incubated with cells in culture. The complexes are thought to stick to the cell surface and as before DNA enters the cells by endocytosis (McCutchan, 1968 and Kawai, 1984). This is the preferred method for transient expression of genes and therefore is not appropriate for the study at hand which required stable transfection of the TP gene for subsequent characterisation.

Electroporation involves the use of high-voltage electric shocks to introduce DNA into cells (Neumann, 1982). Cells are placed in suspension in an appropriate electroporation cuvette. DNA is added and the cells are subjected to a high voltage electrical pulse of defined magnitude and length. The cells are then allowed to recover briefly before they are placed in normal growth medium. This is the method of choice for suspension cultures, as cells must be transferred into cuvettes for transfection.

Liposomal-mediated transfer involves the mixing of DNA with a liposomal suspension comprising cationic lipids and then applying to monolayer cell cultures. Negatively charged phosphate groups on DNA bind to the positively charged surface of the liposome. The residual positive charge is thought to mediate binding to the cell surface (Felgner, 1987) and again DNA enters the cell by endocytosis. This method results in higher efficiency and greater reproducibility than other transfection methods and was the method of choice for the current study.

There are several commercially available liposomal preparations. One such product is the cationic lipid DOTAP (N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate) produced by Boehringer Mannheim (Leventis and Silvius, 1990).

The following chapter describes the transfection of HT-29 colon carcinoma cell lines with TP cDNA inserted into the pcDNA-1-Neo plasmid vector and the control transfections with the vector alone. Subsequent assessment of the cells is described to determine whether increased expression of TP results in alterations in growth kinetics, enzyme expression and most importantly sensitivity to 5-FU.

4.2 Materials and Methods

Firstly, stable HT-29 transfectants were evaluated for their growth kinetics to determine whether transfection with TP would alter the population doubling time of the cells. In addition, the vector alone controls were also assessed to evaluate any effect of transfection alone on growth kinetics.

Secondly, TP protein expression was measured to assess whether the TP transfected cells were expressing increased levels of TP protein compared to the controls. The levels of TS protein were also evaluated by Western immunoblotting. If TS levels were to increase following transfection this may affect 5-FU cytotoxicity and counteract any effects of increased TP.

Thirdly, the MTT growth inhibition assay and the clonogenic assay were used to determine whether increased TP conferred an increased sensitivity to 5-FU *in vitro* with 24 and 72 hours drug exposure times.

Finally, in addition to measuring 5-FU cytotoxicity, inhibition of TS was also quantitated by Western immunoblot analysis. In general, TS inhibition is measured as the amount of ^{3}H-5-FdUMP bound to TS (Moran, 1979 and Priest, 1980). Here an alternative method employs the TS106 monoclonal antibody raised against human TS in a Western immunoblotting assay which distinguishes free TS from that bound in a ternary complex (Drake, 1993). This allows evaluation of TS inhibition in HT-29 transfected cells compared with the vector alone transfected cells and the parental cells.

4.2.1 Geneticin Concentration

Analysis of the role of TP requires cells that contain the TP gene in a stable integrated form. Less than one in every 10^4 cells in a transfection experiment will stably integrate DNA surface (Current Protocols in Molecular Biology, 1997). A dominant selection marker on the plasmid vector is used to isolate stable transfectants. All

plasmid vectors used in the current study contain the Neomycin resistance gene for this purpose. This gene codes for the enzyme aminoglycoside phosphotransferase which detoxifies Neomycin and it's analogues, including Geneticin, that would otherwise inhibit protein synthesis in mammalian cells by interfering with ribosomal function leading to cell death.

Studies were carried out prior to the transfection experiments to determine the appropriate concentration of Geneticin to use for selection. Cells growing in the presence of lethal doses of Geneticin will continue to proliferate through 1-2 growth cycles. As a result, the effects of the drug take several days to manifest themselves (Southern and Berg, 1982).

4.2.1.1 Chemicals and Reagents

DMEM, sodium bicarbonate, L-glutamine, foetal calf serum, penicillin and streptomycin and Trypsin were obtained from Gibco BRL (Paisley, UK). Ham's F10 medium was from SIGMA (Poole, UK), and diaminoethanetetra-acetic acid, disodium salt (EDTA) was obtained from Fisons Scientific Equipment (Loughborough, UK). Geneticin was purchased from SIGMA (Poole, UK).

4.2.1.2 Method

Cells were dissociated from routine culture flasks as described in section 2.2.1.3 and seeded in 100mm petri-dishes at a density of 10^5 cells/mL in 10mLs of 10% serum-containing medium with a range of concentrations of Geneticin from 0.6-1.4mg/mL. Cells incubated in drug-free medium were used as a control. Cells were allowed to grow for 7 days in a humidified atmosphere (2% CO_2 in air) and observed for toxicity.

4.2.2 Transfection of HT-29 cells

4.2.2.1 Chemicals and Reagents

DOTAP Transfection Reagent, N-[1-(2,3-Dioleoyloxy) propyl]-N,N,N-trimethylammonium methylsulfate, was purchased from Boehringer Mannheim (Germany). pcDNA-I-Neo eukaryotic expression vector was purchased from R & D Systems (Abingdon, UK) and the p^{CMV}-TP-Neo vector shown in Figure 4.1 was donated by Dr Roy Bicknell (Molecular Angiogenesis Group, University of Oxford, UK). Plasmid vector pHSG272, was used as a positive control for transfection and was supplied by Dr R. Brown (Department of Medical Oncology, University of Glasgow).

4.2.2.2 Method

HT-29 colon adenocarcinoma cells were dissociated from routine tissue culture flasks as described in section 2.2.1.3. Cells were seeded in 100mm petri dishes at a density of 10^5 cells/mL. After allowing cell attachment and growth to proceed for 24 hours in a humidified atmosphere (2% CO_2 in air), the cells were transfected with the DNA vectors. Three vectors were used: p^{CMV}-TP-Neo containing TP cDNA; pcDNA-I-Neo the vector alone control and pHSG272 as a positive control for transfection, which is known to successfully transfect cells and expresses the G418 resistance gene.

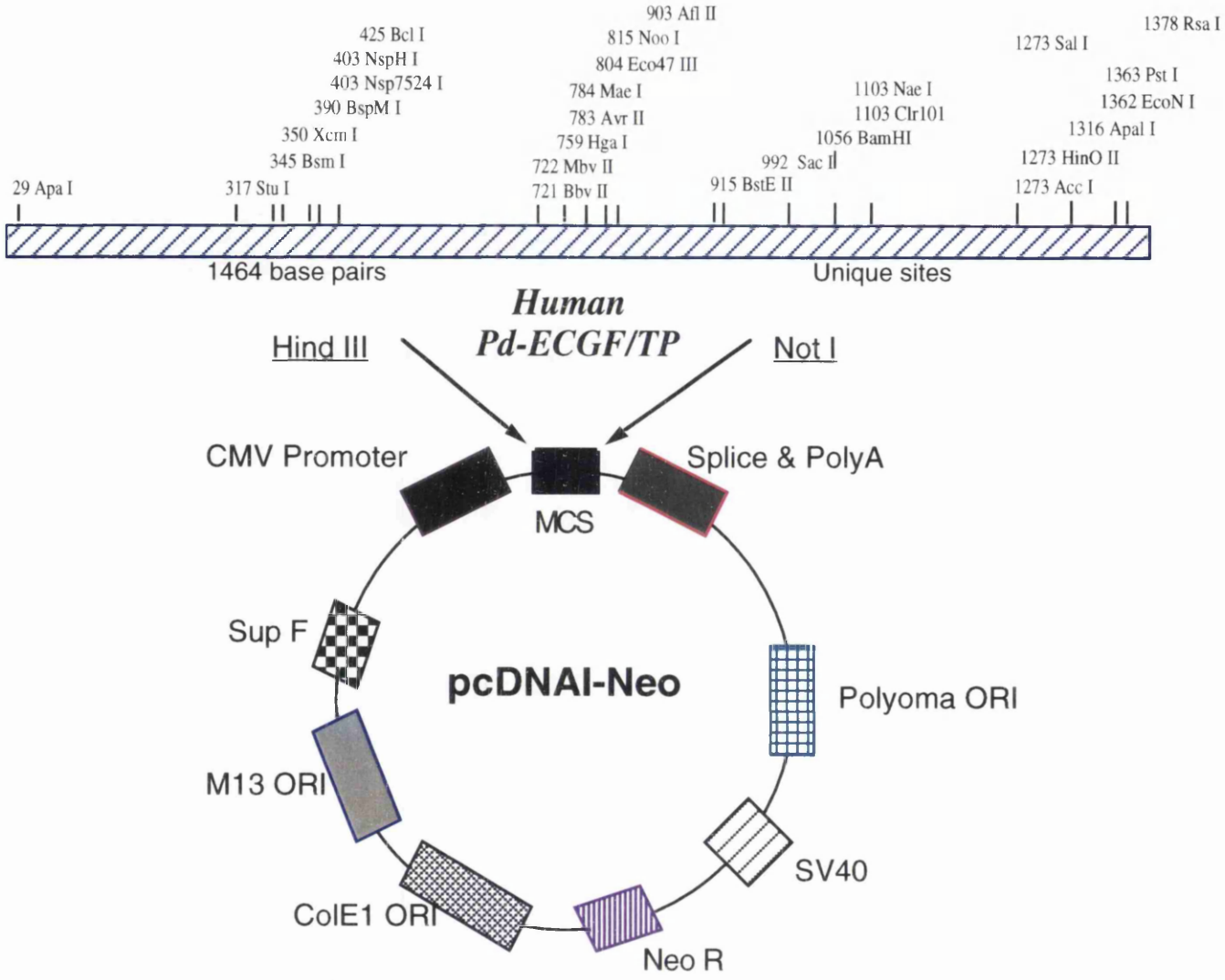
5μg of DNA was diluted in HEPES buffer (20mM) to a final concentration of 0.1μg/mL. In a separate sterile reaction tube, 30μL of the DOTAP preparation was mixed with 70μL of HEPES buffer (20mM). The DNA was then combined with the DOTAP and allowed to stand at room temperature. After 10-15 minutes, the DOTAP/DNA mixture was added to 10mLs of 10% serum-containing medium and the cells were incubated for 16 hours in a humidified atmosphere (2% CO_2 in air). After incubation the medium in the petri dishes was replaced with 10mLs of 10% medium

containing Geneticin (1mg/mL). Cells were then incubated for up to 14 days until colonies started to appear. Selection medium was replenished as required.

4.2.3 Evaluation of effects of transfection

The growth kinetics, TP and TS protein expression and 5-FU sensitivity by MTT and clonogenic assays (sections 2.2.2.2-2.2.4.3) were evaluated in HT-29, HT-29(V) and HT-29(TP) cells to determine the effects of DNA transfection. The cells transfected with pHSG272 were evaluated for growth kinetics and TP expression only, since they were purely a control for transfection.

Figure 4.1 Construction of p^{CMV}-TP-Neo vector. Human Pd-ECGF/TP cDNA was ligated into HindIII and NotI restriction sites of pcDNAI-Neo to create p^{CMV}-TP-Neo was supplied by Dr Roy Bicknell, Molecular Angiogenesis Group, University of Cambridge, UK and pcDNAI-Neo was purchased from R and D Systems, Abington, UK.


903 Afl II
425 Bcl I
815 Noo I
1378 Rsa I
1273 Sal I
403 NspH I
804 Eco47 III
1363 Pst I
403 Nsp7524 I
784 Mae I
1103 Nae I
1362 EcoN I
390 BspM I
783 Avr II
1103 Clr101
1316 ApaI I
350 Xcm I
759 Hga I
1056 BamHI
345 Bsm I
722 Mbv II
992 Sac II
1273 HinO II
29 Apa I
317 Stu I
721 Bbv II
915 BstE II
1273 Acc I
1464 base pairs
Unique sites
Human
Pd-ECGF/TP
Hind III
Not I
CMV Promoter
Splice & PolyA
MCS
Sup F
pcDNAI-Neo
Polyoma ORI
M13 ORI
SV40
ColE1 ORI
Neo R

pCMV-TP-Neo

4.2.4 Measurement of TS inhibition

4.2.4.1 Introduction

TS inhibition is routinely measured as the amount of ^{3}H-5-FdUMP bound to TS (Moran, 1979). Here an alternative method was employed using the TS106 monoclonal antibody raised against human TS. This is a simple method which does not require the use of radioactivity. Free TS and TS bound to FDUP in a ternary complex present in cell lysates are separated by gel electrophoresis, the free TS having a size of 36kDa and TS in the ternary complex having a size of 38.5kDa. Immunoblotting with the TS106 antibody detects both bound and free enzyme (Drake, 1993) and the ratio can be quantified by densitometric scanning.

4.2.4.2 Chemicals and Reagents

See section 2.2.3.1

4.2.4.3 Treatment with 5-FU

Cells were dissociated from routine culture flasks as described in section 2.2.1.3 and seeded in 25cm^2 tissue culture flasks at a density of 2x10^5 cells/mL in 5mLs of 10% serum-containing medium. After allowing cell attachment and growth to proceed for 24 hours in a humidified atmosphere (2% CO_2 in air) the cells were treated with 5-FU at IC_{10} and IC_{50} concentrations (5 and 10μM respectively) for 1 and 24 hours. Drug-free control flasks were also set up and lysates prepared at identical time points as described above.

4.2.4.4 Cell lysate preparation

See section 2.3.2.2

4.2.4.5 Electrophoresis

See section 2.3.2.4

4.2.4.6 Immunoblotting

See section 2.3.2.5

The ratio of bound to free TS was quantitated by densitometric scanning of the film.

4.2.5 Statistical Analysis

Analysis of Variance (the F test) was used to identify differences in the sensitivities of HT-29, HT-29(V) and HT-29(TP) cell lines to 5-FU. Students' t-tests were used to determine the significance of the difference identified.

4.3 Results

4.3.1 Determination of G418 concentration

The concentration of the selection agent G418 used for transfection experiments was determined as the minimum concentration required to effect 100% cell kill as determined visually by a reduction in colony numbers in 7 days and was 1mg/ml.

4.3.2 Transfection using DOTAP

HT-29 cells were transfected with the vectors pcDNA-I-Neo, p^{CMV}-TP-Neo and pHSG272 using the cationic liposomes DOTAP. Cells were grown in selection medium containing G418 (1mg/mL). A minimum of 50 surviving colonies were pooled from each transfection experiment.

4.3.3 Growth Kinetics

The growth kinetics of the HT-29(TP), the HT-29(V) cells and the parental cells, HT-29, were assessed. Growth curves are illustrated in Figure 4.2 and the population

doubling times are shown in Table 4.1. The population doubling time was not altered in the HT-29(TP) cells compared with the HT-29(V) and the parental cells.

4.3.4 TP protein expression

TP protein expression was evaluated by Western immunoblot analysis, in order to verify that the HT-29(TP) cells express an increased level of TP protein compared to the controls. The Western immunoblot is shown in Figure 4.3. As described in section 2.3.2, TP protein was not detected in the HT-29 parental cells. TP protein was also undetected in the vector alone controls and the cells transfected with the control vector pHSG272. TP protein was however detected in the cells transfected with 5μg of TP cDNA, and to a lesser extent in those transfected with 10μg of TP cDNA. The transfection experiments were therefore successful, resulting in increased expression of TP protein in HT-29(TP) cells.

4.3.5 TS Protein Expression

TS protein expression was evaluated in the HT-29(TP), HT-29(V) and HT-29 parental cells by Western immunoblot analysis with the TS106 antibody. As can be seen in Figure 4.4 there was no change in TS protein expression in the three cell lines. Transfection with TP cDNA or transfection alone did not, therefore, alter the expression of one of the target enzymes for 5-FU.

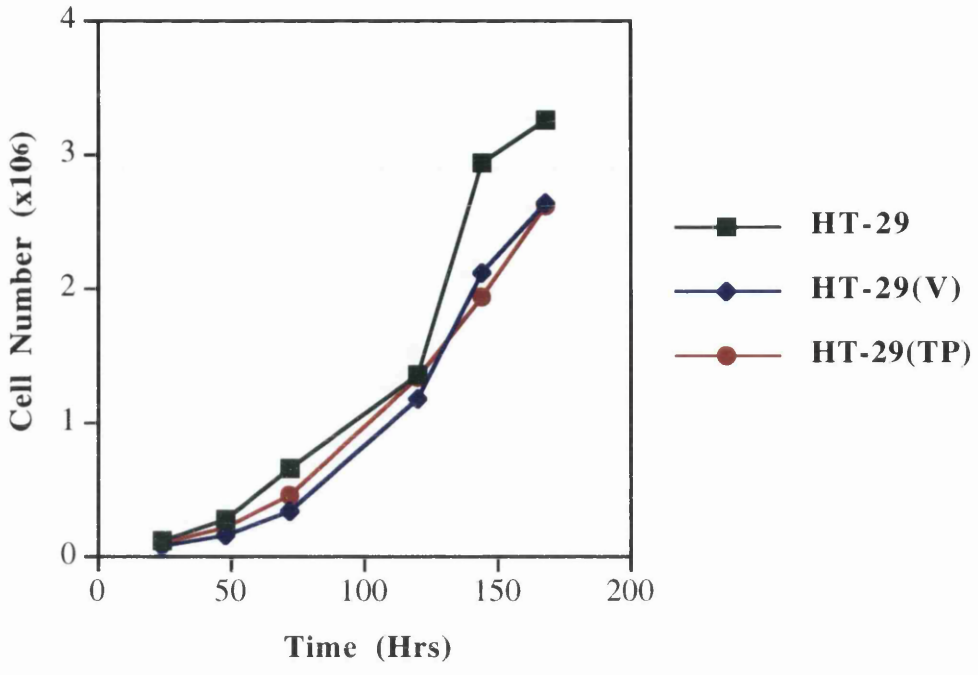


Figure 4.2 Growth curves of HT-29, HT-29(V) and HT-29(TP) cell lines. Points are means and standard deviations of 3 counts carried out daily.

Cell Line	Population Doubling Time(Hrs)
HT-29	30
HT-29(V)	30
HT-29(TP)	30.5

Table 4.1 Population doubling times of HT-29 parental cells, the vector alone controls; HT-29(V) and the TP transfected cells; HT-29(TP)

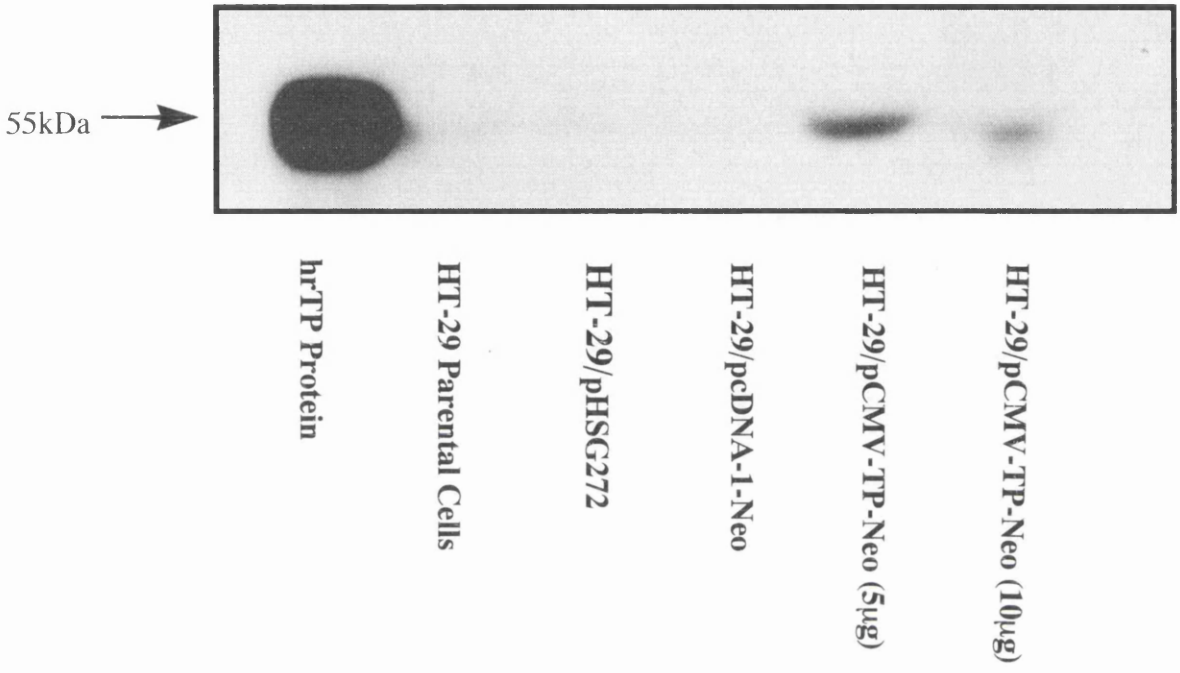


Figure 4.3 TP Western immunoblot using PGF.44c anti-TP antibody. From left to right, human recombinant TP protein (hrTP), HT-29 parental cells, HT-29 cells transfected with pHSG272, pcDNA-I-Neo and p^{CMV}-TP-Neo (pcDNA-I-Neo containing TP cDNA) 5 and 10μg. For analysis of TP protein levels, cells were lysed and cytosols prepared by centrifugation at 10,000g. 100μg of cytosol was size-fractionated by SDS-polyacrylamide gel elctrophoresis. Proteins were electroblotted onto nitrocellulose membranes and incubated with PGF.44c, a mouse monoclonal antibody raised against human TP, followed by an anti-mouse secondary antibody linked to HRP. Protein-antibody complexes were visualised using enhanced chemiluminescence (see secions 2.2.3.2-2.2.3.5).

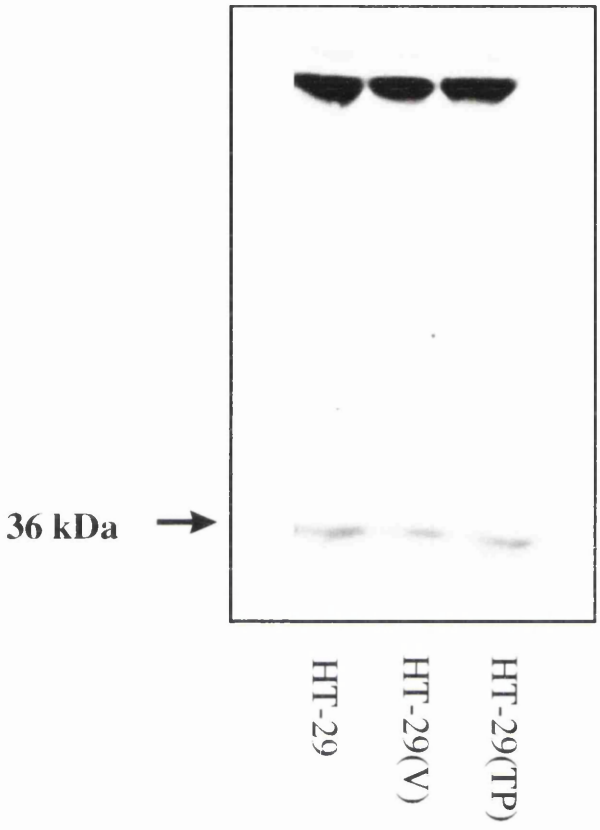


Figure 4.4 TS Western immunoblot using TS 106 anti-TS monoclonal antibody. HT-29 parental cells, HT-29 cells transfected with the vector alone, HT-29(V) HT-29 transfected with TP, HT-29(TP). Transfected cells were selected by long-term incubation with Geneticin. For analysis of TS protein levels, cells were lysed and cytosols prepared by centrifugation at 10,000g. 100µg of cytosol was size-fractionated by SDS-polyacrylamide gel electrophoresis. Proteins were electroblotted onto nitrocellulose membranes and incubated with TS 106, a mouse monoclonal antibody raised against human TS, followed by an anti-mouse secondary antibody linked to HRP. Protein-antibody complexes were visualised using enhanced chemiluminescence.

4.3.6 5-FU Cytotoxicity

The transfected cells were assessed for their sensitivity to 5-FU using the growth inhibition assay and the clonogenic assay. Cells were exposed to 5-FU for 24 and 72 hours. Representative dose response curves for both the MTT and clonogenic assays are shown in Figures 4.5 and 4.6 respectively. The corresponding 5-FU IC_{50} values are outlined in Table 4.2.

	5-FU IC_{50} (μM)		
Cell Line	**Growth Inhibition (MTT) Assay**		**Clonogenic Assay**
	24 hours (n=30)	**72 hours (n=6)**	**24 hours (n=3)**
HT-29	9.8±5.8	3.9±0.3	17.7±8.1
HT-29(V)	14.5±2.0	3.9±0.9	20.3±19.4
HT-29(TP)	8.8±0.6	4.5±0.2	13.5±6.7

Table 4.2 Mean 5-FU IC_{50}'s ± standard errors (SE) determined by the Growth Inhibition(MTT) Assay and the Clonogenic Assay in HT-29, HT-29(V) and HT-29(TP) cells.

4.3.7 Statistical Analysis

There was an 8.7-fold range in 5-FU IC_{50} (3.3-28.8μM) obtained for HT-29 cells as measured by the MTT assay (n=30) following a 24 hour exposure to 5-FU (CV=42.5%). A single cytotoxicity experiment involved triplicate plates for each cell line with at least 4-8 wells within each plate for each drug concentration. Analysis of Variance of the IC_{50}'s obtained for HT-29, HT-29(V) and HT-29(TP) demonstrated that, despite the apparent variability in IC_{50}s from one week to another, none of the experiments gave significantly different results ($F = 1.184$, $p > 0.05$, $n = 90$). In other words the difference in sensitivities observed was between cell lines and not between experiments, since the same order of sensitivity was observed from one experiment to another.

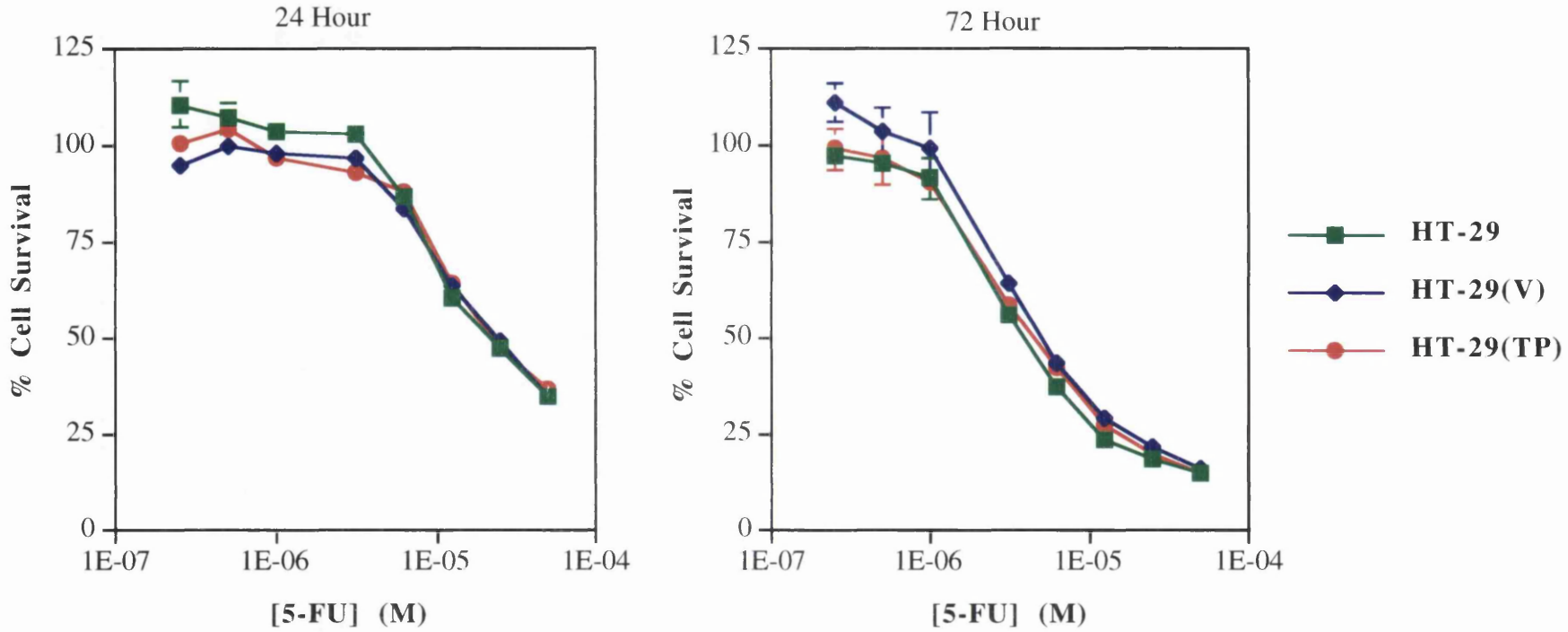


Figure 4.5 Representative dose-response curves for 5-FU cytotoxicity measured by the MTT assay in HT-29(TP) cells compared with HT-29(V) and the parental cells HT-29 cells. Growth inhibition was measured in 96-well plates, (500 cells/well in Ham's F10/DMEM medium containing foetal bovine serum). Increasing concentrations of 5-FU were added in medium for 24 or 72 hours after allowing cells to attach and grow for 48 hours. Cell growth was measured by incubation with MTT (50µg/mL) for 4 hours following a period of recovery in serum-containing medium (drug-free). Error bars represent standard error of means of three 96-well plates, with 4 wells per plate. IC50 values are outlined in Table 4.2.

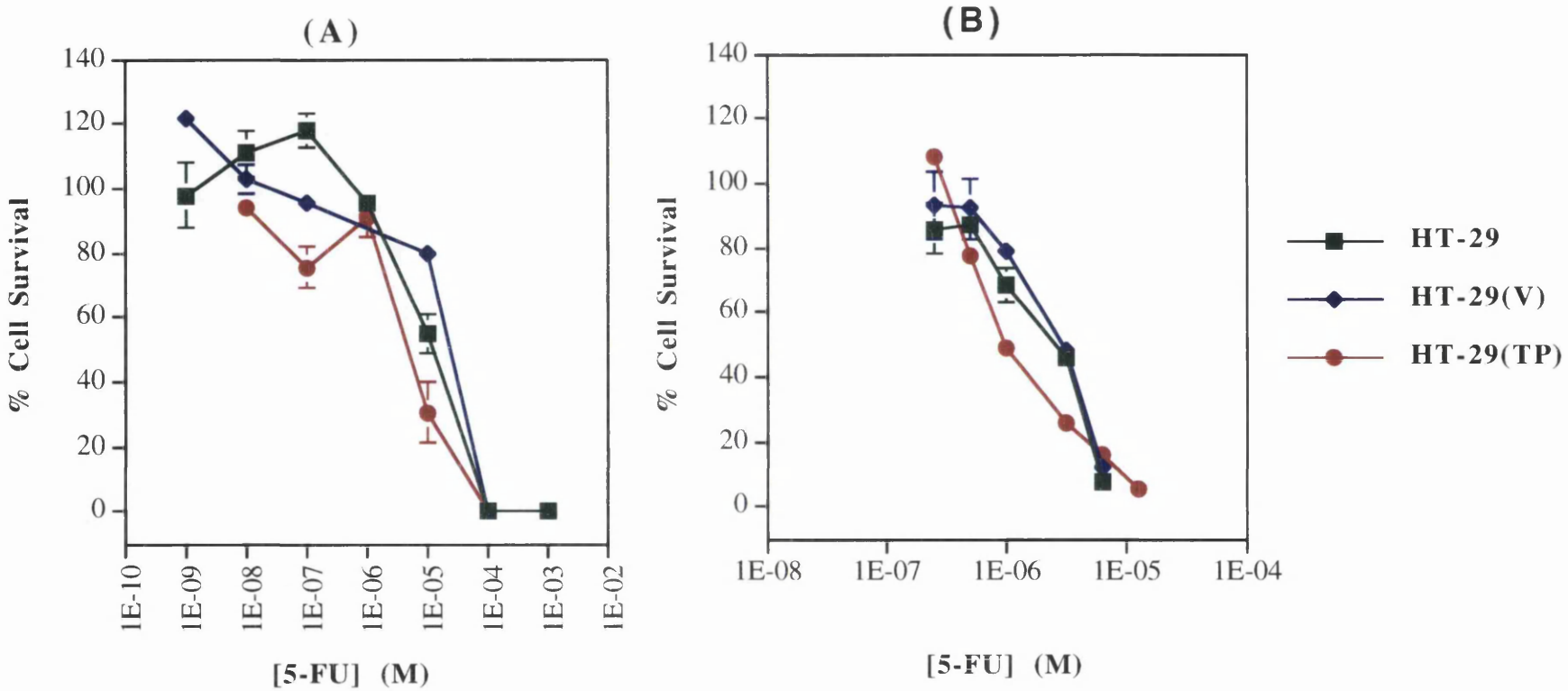


Figure 4.6 Representative dose-response curves for 5-FU cytotoxicity (24 (A) and 72 (B) hours) measured by the clonogenic assay in HT-29(TP) cells compared with HT-29(V) and the parental cells HT-29 cells. Cells were seeded into 25cm^2 culture flasks in 5mls of 10% serum containing medium and allowed to adhere and grow for 48 hours. Cells were then treated with increasing concentrations of 5-FU for 24 hours, (one flask per drug concentration). Following drug treatment, control untreated cells were serially diluted giving a final concentration of 10^3 cells/ml and 1ml was added to 4 x 50mm petri-dishes along with 4mls of medium giving a final concentration of $2x10^2$ cells/ml. Drug treated cells were then serially diluted in the same manner as the controls and dishes were incubated at 37°C, 2% CO_2 in a humidified atmosphere for 10 days. Colonies were fixed, stained and counted and cell survival was measured as a percentage of the control counts. 5-FU IC_{50} values are outlined in Table 4.2.

When comparing 5-FU cytotoxicity between HT-29, HT-29(V) and HT-29(TP) cell lines using Analysis of Variance, a significant difference in their sensitivity was observed ($F= 6.432$, $p<0.005$, $n=30$). On further analysis HT-29(TP) cells were shown to be significantly more sensitive to 5-FU than the HT-29(V) cells ($p<0.005$, Students' t-test). This is demonstrated by a 1.7-fold decrease in 5-FU IC_{50}. There was however no significant difference between HT-29(TP) and HT-29 cells ($p>0.01$, Students' t-test). The results obtained from the clonogenic assay were more variable than the MTT assay with larger standard errors. There is, however, a trend for HT-29(TP) cells to be more sensitive than HT-39(V).

4.3.8 TS Inhibition

Inhibition of TS was assessed by Western immunoblot analysis of HT-29(TP), HT-29(V) and HT-29 cells treated with 5-FU at IC_{10} and IC_{50} concentrations for one and 24 hours. Drug-free controls were also assessed at 1 and 24 hours. Immunoblots are illustrated in Figures 4.7 and 4.8, representing cells treated with 5-FU IC_{10} and IC_{50} concentrations respectively.

As can be seen in both Figures 4.7 and 4.8 there was no ternary complex (38kDa) detected following 1 hour treatment with 5-FU in HT-29, HT-29(V) and HT-29(TP) cells (lanes 2, 6 and 10 respectively). After 24 hours in the presence of drug, an additional band was apparent, approximately 38kDa in size (lanes 4, 8 and 12) which was not present in the drug-free controls. This corresponds to TS bound in a covalent ternary complex with 5-FdUMP and reduced folates (Johnston, 1991). Densitometric scanning of these bands showed that levels of bound TS were higher in HT-29(V) and HT-29(TP) cells than in the HT-29 controls (Tables 4.3 and 4.4). Free TS (36kDa) levels were unchanged following 1 hour drug treatment compared with the drug-free controls for each cell line (comparing lanes 2, 6 and 10 with 1, 5 and 9 respectively).

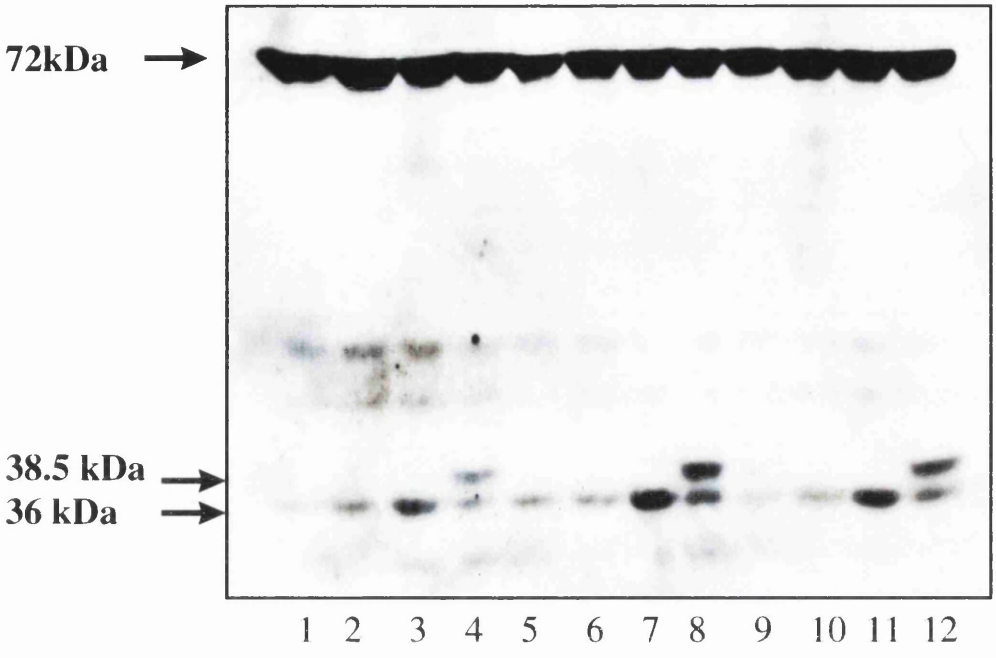
However, free TS was also increased after 24 hours exposure in HT-29(V) and HT-29(TP) cells, thus the ratio of bound to free TS was unchanged, as shown in Tables 4.3 and 4.4.

Cell Line	OD x mm		
	5-FU (5μM) / 24 hours		
	Free TS	Bound TS (Ternary complex)	TS Ratio Bound : Free
HT-29	0.6	0.8	1.3
HT-29(V)	2.2	2.6	1.2
HT-29(TP)	1.8	3.7	1.7

Table 4.3 (OD x mm) measurements of Western immunoblot of HT-29, HT-29(V) and HT-29(TP) cell lines following treatment with 5μM 5-FU (Figure 4.7).

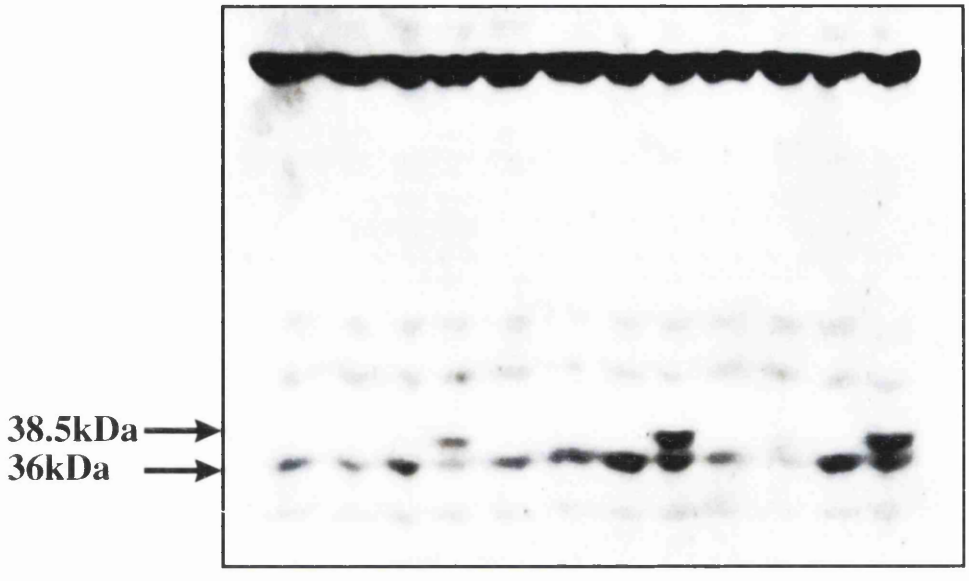
Cell Line	OD x mm		
	5-FU (10μM) / 24 hours		
	Free TS	Bound TS (Ternary complex)	TS Ratio Bound : Free
HT-29	0.7	0.9	1.2
HT-29(V)	1.5	1.8	1.2
HT-29(TP)	1.1	1.5	1.4

Table 4.4 (OD x mm) measurements of Western immunoblot of HT-29, HT-29(V) and HT-29(TP) cell lines following treatment with 10μM 5-FU (Figure 4.8).



Cell line	HT-29				HT-29(V)				HT-29(TP)			
5-FU	-	+	-	+	-	+	-	+	-	+	-	+
Duration	1	1	24	24	1	1	24	24	1	1	24	24

Figure 4.7 TS Western immunoblot of HT-29, HT-29(V) and HT-29(TP) cells following treatment with 5-FU (5μM) for 1 and 24 hours. Cells were lysed and cytosols prepared by centrifugation at 10,000g. TS bound in a ternary complex with FdUMP and reduced folates was distinguished from free TS by size, 38.5 and 36kDa respectively. 100μg of cytosol was size-fractionated by SDS-polyacrylamide gel electrophoresis. Proteins were electroblotted onto nitrocellulose membranes and incubated with TS 106, a mouse monoclonal antibody raised against human TS, followed by an anti-mouse secondary antibody linked to HRP. Protein-antibody complexes were visualised using enhanced chemiluminescenc.



Cell line	HT-29				HT-29(V)				HT-29(TP)			
5-FU	-	+	-	+	-	+	-	+	-	+	-	+
Duration	1	1	24	24	1	1	24	24	1	1	24	24

Figure 4.8 TS Western immunoblot of HT-29, HT-29(V) and HT-29(TP) cells following treatment with 5-FU (10μM) for 1 and 24 hours. Cells were lysed and cytosols prepared by centrifugation at 10,000g. TS bound in a ternary complex with FdUMP and reduced folates was distinguished from free TS by size, 38.5 and 36kDa respectively. 100μg of cytosol was size-fractionated by SDS-polyacrylamide gel electrophoresis. Proteins were electroblotted onto nitrocellulose membranes and incubated with TS 106, a mouse monoclonal antibody raised against human TS, followed by an anti-mouse secondary antibody linked to HRP. Protein-antibody complexes were visualised using enhanced chemiluminescence.

4.4 Discussion

The aim of this study was to transfect HT-29 human colon carcinoma cells with the gene encoding TP and to assess it's contribution to 5-FU cytotoxicity and potential gene therapies. In order to assess this effect it was first necessary to confirm that transfection had occurred and then to establish whether this altered the growth kinetics of the cells or the expression of the target enzyme.

4.4.1 DNA Transfection

Cells were successfully transfected with TP cDNA. This was confirmed by an increase in TP protein expression detected by Western immunoblotting. Two concentrations of DNA were used in the transfection experiments (5 and 10μg). The levels of TP protein detected in transfections using 10μg of p^{CMV}-TP-Neo, although greater than the parental cells, were less than the transfections using 5μg of DNA. This may be related to the mechanism by which cationic liposomes carry DNA and allow it's entrance into the cell. The increased quantity of DNA in the 10μg transfection experiments may have resulted in saturation of the positive charges on the cationic liposomes, leaving no or few residual positive charges for binding to the negatively charged sialic acid residues on the cell surface (Current Protocols in Molecular Biology, 1997). Since the 5μg transfection was more successful, these cells were used for subsequent experiments.

Transfection with TP had no effect on the growth characteristics of the cells and there was no difference in the basal levels of the target enzyme TS compared to the pcDNA-1-Neo transfectants and parental cells. Therefore, any changes in the sensitivity of the cells to 5-FU cannot be attributed to these factors.

4.4.2 Cell Sensitivity to 5-FU

Having established an increase in TP protein expression in the HT-29(TP) cells, together with unaltered growth kinetics and TS levels, the potential contribution to 5-FU sensitivity was assessed using MTT and clonogenic cytotoxicity assays. There was a striking 8.7-fold variation in 5-FU IC_{50}'s obtained for HT-29 cells alone after multiple experiments (range 3.3-28.8μM, CV=42.5%) when measured by the MTT assay following a 24 hour exposure to 5-FU. Each cytotoxicity experiment involved triplicate plates with at least 4 wells within each plate for each drug concentration. It may be that the variability is due to factors such as pipetting, culture media and drug concentration. For example, new drug stock was weighed and dissolved in PBS for each experiment. Drug solutions were then filter sterilised and non-specific binding to the filters may have varied from one experiment to another. For the purposes of this study, however, it is of more value to consider the relative difference in 5-FU IC_{50} between cell lines within each experiment.

In comparing 5-FU cytotoxicity (using a 24 hour exposure time) between cell lines using the MTT assay, there is a small increase (1.6-fold) in the sensitivity of HT-29(TP) cells to 5-FU compared to the vector only controls. Although this increase is small there is a significant difference ($p<0.001$, Students' t-test) in the mean IC_{50}s between these cell lines following 24 hour treatment. There is however no difference in 5-FU IC_{50} between the parental and HT-29(TP) cells ($p>0.1$, Students' t-test). The clonogenic assay confirmed that there was no difference between the parental cells and the HT-29(TP) line. It was not possible to confirm the increased sensitivity of the HT-29(TP) cells compared to the HT-29(V) cells using the clonogenic assay since there was a larger variation in the IC_{50}'s obtained for the HT-29(V) cells.

It would appear, therefore, that the presence of the TP gene does increase the sensitivity of the cells to 5-FU. The presence of the vector alone appears to have resulted in increased resistance to 5-FU following 24-hour treatment and this cannot be attributed to altered basal levels of TS. The further inclusion of the gene encoding TP may have resulted in sensitisation of the cells to a similar level as that of the parental cells. It is important to note that both HT-29(V) and HT-29(TP) cell lines are pools of transfected cells and therefore the apparent differences in 5-FU sensitivity are not due to clonal variations.

4.4.3 TS Inhibition

Resistance to 5-FU can be attributed to increased levels of TS (Berger, 1987 and Clark, 1987) and this may mask the effects of any increase in TP activity. In contrast, decreased TS levels may exaggerate any effects caused by increased TP activity. Indeed, the present study has shown that TS protein expression does correlate with 5-FU IC_{50} in a number of colon cell lines (Figure 2.7). In the current study, however, transfection of TP did not lead to altered basal levels of TS or growth kinetics (Figures 4.4 and 4.2 respectively). Therefore, any change in sensitivity of the transfected cells to 5-FU could not be explained by either of these factors.

Inhibition of the target enzyme TS was investigated in the HT-29, HT-29(V) and HT-29(TP) cells following treatment with 5 and 10μM 5-FU. There was no measurable change in the level of TS inhibition in the 3 cell lines. Basal levels of TS increased with time in culture from 1 to 24 hours in all cells analysed. This is due to cell proliferation and an increased requirement for DNA synthesis (Heidelberger, 1975, Conrad and Ruddle, 1972 and Pestalozzi, 1995).

5-FU treatment resulted in an increase in total TS levels following exposure to 5 and 10μM 5-FU in all cell lines compared with drug-free controls. This is thought to be

a protective mechanism of cells in response to cytotoxic stress, especially from agents which bind to TS and result in changes in the metabolite pools, most significantly the depletion of thymidylate (Pestalozzi, 1995 and Washtein, 1983). Interestingly, total TS protein levels (bound and free) were higher in both the HT-29(V) and HT-29(TP) cells compared to the parental cells following exposure to 5-FU for 24 hours. The reason for this is unclear. It does however, confirm that transfection alone has resulted in biochemical alterations in cells as seen with the cytotoxicity studies where HT-29(V) cells were more resistant to 5-FU compared to the parental cells. As a result of the effect of time and 5-FU treatment on TS, interaction between drug and target enzyme is best evaluated by studying the free/bound drug ratio.

There was no measurable change in the ratio of bound to free TS in the 3 cell lines, however the increase in sensitivity of the HT-29(TP) cells compared to the HT-29(V) was small (1.6-fold) and it may be that this small difference could not be detected by Western blot analysis.

Although it is clear that TS inhibition is occurring in these cells, it is not apparent whether this is the principle mechanism of action of 5-FU. It may be that incorporation into RNA or DNA is also important. Spears and colleagues reported that tumour sensitivity in murine tumour models is associated with complete inhibition of TS activity (Spears, 1982). This was not achieved in the current study at the time points analysed, which may suggest that TS inhibition is not the principle mechanism of action. However, further experiments with a wider range of time-points would be required to draw any conclusions as to the role of TS inhibition.

In summary, this study has demonstrated that HT-29 colon cancer cell lines can be transfected with TP without altering growth kinetics or basal expression of TS. HT-29(TP) cells were more sensitive to 5-FU than the vector alone controls suggesting that TP plays a role in 5-FU cytotoxicity. There was however no difference in sensitivity of

TP transfected and parental cells nor any evidence of altered TS inhibition. The effect of TP on sensitivity to 5-FU may, therefore, be mediated by mechanisms other than TS.

4.4.4 Increased TP activity in other cell lines

Other groups have sought to determine the role of an increase in TP activity in colon and other tumour cell lines using DNA transfection techniques. The first study was carried out by Haraguchi *et al* who transfected human KB epidermal carcinoma cells with TP resulting in a clone with high TP activity (KPE-3) compared to the parental cells (KB) which have no endogenous TP activity (Haraguchi, 1993). There was no significant difference in the sensitivity of the KPE-3 cells to 5-FU compared to the parental cells. The effects of 5-FU were not reversed by thymidine, indicating that TS inhibition is not the principle mechanism of action of 5-FU in these cells and therefore these cells were not a good model for this investigation. KPE-3 cells were however more sensitive to the 5-FU pro-drugs 5'-DFUR (19-fold) and Tegafur (2.3-fold) than 5-FU. Despite this, overall growth inhibition was no greater with 5'-DFUR than with 5-FU alone (IC_{50} 13.3±2.8 and 7.0±1.0 respectively). Increased TP activity had therefore resulted only in an increased capacity of the cells to convert 5'-DFUR to 5-FU.

In another study, MCF-7 breast adenocarcinoma cells with low basal levels of TP activity were transfected with TP (Patterson, 1995). Two clones were subsequently characterised, one with 100-fold increased TP activity (TP4) and the other with a 7-fold increase (TP7). There was no difference in the sensitivity of TP4 or TP7 cells to 5-FU compared to the MCF-7 parental cells. Exogenously added thymidine did not reverse 5'-DFUR cytotoxicity in the parental MCF-7 cells suggesting that in this cell line 5-FU cytotoxicity is not TS related. Cytotoxicity was partially reversed by thymidine in the clone with 100-fold increased TP activity suggesting the emergence of a minor role for TS inhibition in the cells. Increased TP activity enhanced the sensitivity of TP4 and TP7

cells to 5'-DFUR compared with the controls (IC_{50}'s 0.104, 7.1 and 17.33μM respectively). However, with a 4-fold increase in TP in the TP4 clone, these cells were still less sensitive to 5'-DFUR than 5-FU. Only when TP was increased 100-fold (TP7 cells) was 5'-DFUR more cytotoxic than 5-FU (IC_{50}'s of 1.44±0.96μM and 7.1±1.7μM for 5'-DFUR and 5-FU respectively). Again increased TP activity had resulted only in an increased capacity of the cells to convert 5'-DFUR to 5-FU and had no effect on 5-FU cytotoxicity itself.

Transfection of PC-9 lung adenocarcinoma cells with TP resulted in cells with 50-fold greater TP activity (PC9-DPE2) compared with vector alone controls and parental cells (Kato, 1997). PC-9-DPE2 cells were 8-fold more sensitive to 5-FU than controls. They were also more sensitive to the 5-FU pro-drugs Tegafur and 5'-DFUR (26- and 167-fold decrease in IC_{50} respectively). Reversal of TS inhibition with thymidine was not investigated nor were the levels of 5-FU nucleotide anabolites or incorporation into RNA or DNA. It is therefore not possible to postulate the mechanism of action of 5-FU in these cells.

These experiments cast doubt on the importance of TP in relation to 5-FU cytotoxicity. The best evidence for a role of TP in 5-FU cytotoxicity came from Schwartz *et al* who demonstrated that IFN-α enhanced the cytotoxicity of 5-FU through an increase in TP activity, in HT-29 cells (Schwartz, 1992). Transfection studies with TP cDNA resulted in 5 clones with 1.8- to 5.4-fold greater TP activity than the parental cells which were characterised for their relative sensitivity to the fluoropyrimidine 5-FU (Schwartz, 1995). All clones demonstrated greater sensitivity to 5-FU than the parental cells, ranging from a 2- to 5-fold decrease in 5-FU IC_{50} in four clones and a 19-fold decrease in IC_{50} was observed in the clone with a 5.4-fold greater TP activity.

In these experiments by Schwartz *et al* there was a significant correlation (r=0.9) between the relative increase in TP activity and the relative decrease in 5-FU IC_{50}. There

was also an associated increase in 5-FdUMP levels in the transfected cells following 5-FU exposure. Schwartz *et al* compared the results from the IFN-α studies with that of the DNA transfection studies and found that in both cases increased TP activity correlated with increased levels of 5-FdUMP and most importantly increased sensitivity to 5-FU. TS activity decreased following transfection compared with the parental cells. This reduction in target enzyme activity may have contributed in part to the increase in sensitivity to 5-FU.

The present study used the same DNA construct and HT-29 colon adenocarcinoma cells as that used by Schwartz *et al.* The impact of increased TP expression on 5-FU cytotoxicity differed however between the two studies. The IC_{50} of 5-FU in Schwartz's HT-29 cells was 2.53±0.74μM following treatment with 5-FU for seven days compared with 9.9±5.8μM and 3.9±0.3μM for cells treated with 5-FU for 24 and 72 hours in the current study. It is not possible to compare 5-FU cytotoxicity in Schwartz's HT-29 cells and the cells used in the present study since drug exposure times were different. There are however several differences in the methods employed by Schwartz compared with the present study. Schwartz dosed cells for 7 days in the same drug/medium solution. By contrast in the current study, cells were dosed with 5-FU for either 24 hours followed by 72 hours of daily replenishment of medium or 72 hours with daily dosing of drug and a similar recovery period.

Another factor which might explain, in part, the difference in results is the methods employed for measuring 5-FU cytotoxicity. The cytotoxicity assay used by Schwartz and colleagues measured total protein concentration by Sulforhodamine B (SRB) staining of cells treated with 5-FU compared with untreated cells to determine growth inhibition. It does not distinguish between living and dead cells or proliferating and non-proliferating cells. The methods used in the present study evaluated alternative functions of the cell as a measure of cytotoxicity. The MTT assay is similar to the SRB

assay but can distinguish between living and dead cells. The clonogenic assay measures the ability of individual cells to proliferate after drug exposure. In addition, drug treatment in the SRB assay differed from the MTT assay used as described above. Although these differences may lead to differences in IC_{50}'s, the relative sensitivity between transfected and parental cells should be consistent if increased TP expression did confer greater sensitivity to 5-FU. Therefore these differences remain unexplained.

4.4.5 Summary

There is considerable variation in the effects of TP transfection on 5-FU sensitivity in different cell lines. It is worth noting that in those studies in which no increase in sensitivity to 5-FU was reported there was no reversal of 5-FU cytotoxicity by thymidine in the parental cells. This suggests that TS inhibition is not the principle mechanism of action of 5-FU in these cells and increasing TP activity has no impact. These cells may utilise other routes of 5-FU cytotoxicity. For example Kufe and colleagues showed that RNA toxicity is the principle mechanism of action of 5-FU in MCF-7 cells (Kufe, 1981). By contrast, Schwartz *et al* showed that TP transfection did confer sensitivity to 5-FU in a cell line where TS expression was reduced (Schwartz, 1995). Schwartz and colleagues demonstrated that TS inhibition was an important mechanism of cytotoxicity for HT-29 cells, illustrated by an increased inhibition following transfection with TP. Kato and colleagues demonstrated increased 5-FU sensitivity in TP transfected cells but reversal by thymidine was not investigated therefore no comment can be made regarding the principle mechanism of cytotoxicity (Kato, 1997).

In the present study, TS inhibition has been observed, however it's contribution to the overall cytotoxicity of 5-FU is not certain. If screening of cell lines for thymidine-

mediated reversal of 5-FU cytotoxicity had been included in the characterisation of cell lines, selection of a more relevant model to test the hypothesis may have been ensured.

This study has demonstrated an increase in TP protein expression and 5-FU cytotoxicity in HT-29 colon carcinoma cells transfected with TP cDNA compared to vector alone controls. No change in 5-FU cytotoxicity was observed between HT-29 and HT-29(TP) cells however. This increase in TP expression did appear to contribute to 5-FU sensitivity when measured using the growth inhibition (MTT) assay, following 24 hour drug exposure. No difference was observed following 72-hour treatment. There was little change in the ratio of TS bound in a ternary complex to free TS enzyme between HT-29, HT-29(V) and HT-29(TP) cells as measured by Western immunoblotting following treatment of cells with IC_{10} and IC_{50} concentrations of 5-FU.

It may be that there are other rate-limiting factors which when addressed may result in a greater increase in sensitivity of the HT-29(TP) cells to 5-FU. For example, reduced folate concentrations may be limiting TS activity/inhibition. This will be addressed in Chapter 6 along with other potentially rate-limiting factors such as deoxyribose-1-phosphate (the co-substrate for TP). The ability of thymidine to rescue HT-29(TP) cells from 5-FU will also be assessed in this chapter to assess whether the inhibition of TS is important for 5-FU cytotoxicity in HT-29 cells. Finally, despite an increase in TP protein expression, in the transfected cells an increase in TP activity must be confirmed. This is discussed in the next chapter.

CHAPTER 5

5. TP Activity Assay

5.1 Introduction

Having established increased TP protein expression in the HT-29(TP) cells which resulted in a small increase in 5-FU cytotoxicity compared with the vector alone controls, the primary purpose of this study was to ensure that the observed increase in TP protein reflected an increase in enzyme activity. An enzyme assay was therefore developed to measure the activity of thymidine phosphorylase (TP) *in vitro*. This assay was subsequently used to place TP activity in HT-29 cells in context with other colon carcinoma cell lines and also cell lines derived from other tumour types. Enzyme activity in cell lines was then compared to that of normal colon mucosal tissue and colon tumours.

TP catalyses the reversible phosphorolysis of thymidine, deoxyuridine and it's analogues to their respective bases and deoxyribose-1-phosphate, Reaction 1 below (Zimmerman and Seidenberg, 1964; Krenitsky, 1967; 1981 and Schwartz, 1971). TP also catalyses a deoxyribosyl transfer from one deoxynucleotide to another base to form a second deoxynucleotide, Reaction 2 below (Zimmerman, 1964; Zimmerman and Seidenberg, 1964; Gallo, 1967 and Krenitsky, 1967)

Reaction 1: Thymidine + $Phosphate_i$ $\rightleftharpoons$ Thymine + dR-1-P

Reaction 2: Thymine* + Thymidine $\rightleftharpoons$ Thymidine* + Thymine

Many currently used methods for the measurement of TP activity utilise the former reaction i.e., thymidine to thymine (Schwartz, 1971 and Iltzsch, 1985). For the purposes of this study however, a method has been developed to measure the reverse of this reaction and uses 5-FU in place of thymine as the substrate. TP catalyses the conversion of 5-FU to 5-FdUrd and this reaction is the focus of the study.

Two enzyme assay methods have been previously described: a spectrophotometric method and a thin layer chromatography (TLC) method. The spectrophotometric assay is a modification of the method developed by Friedkin and Roberts (1953) and has been used in a number of studies (Moghaddam, 1992; Heldin, 1993; Miyadera, 1995; Patterson, 1995; Sumizawa, 1993; Haraguchi, 1993 and Yoshimura, 1990). This assay is based on the difference in molar extinction co-efficients between thymidine and thymine at alkaline pH. Thymidine is incubated with enzyme preparation (e.g. purified enzyme or crude cell lysate) and tris buffer, pH 7.5 at 37°C. The formation of thymine can then be measured at 300nm on a spectrophotometer. One measurement is made at a fixed time point of 90 minutes (Friedkin and Roberts, 1953) or at 16 hours (Patterson, 1995). This assay appears not to be fully validated. In few studies has the linearity of the assay with respect to time, protein or substrate concentration been evaluated. In addition, contaminating substances in crude enzyme preparations interfere with this assay, making it difficult to determine TP's activity. An improvement in the assay is observed after purification of the enzyme.

The second method is based on both thin-layer chromatography and radiometric analysis. This method was developed by Gan (1981) and was developed further by Yoshimura and colleagues (1990) and involves the use of [^{14}C]-labelled thymidine or thymine. This assay has been used in a number of studies (Kouni, 1993; Kato, 1997 and Miwa, 1986). TP activity can be assayed either by the conversion of [^{14}C]-thymidine to [^{14}C]-thymine or *vice versa*. Incubations contain substrate (radiolabeled thymine or thymidine), buffer, enzyme preparation/cell lysate and sodium phosphate or 2-deoxyribose-1-phosphate respectively depending on the substrate used. After 15-45 minutes the reaction is terminated by boiling and samples are spotted onto chromatography paper or plates and developed by ascending chromatography. Bands corresponding to thymidine and thymine are detected using an ultra-violet (UV) lamp

and cut out of the chromatography paper or scraped off the plates and the radioactivity is quantified using a scintillation counter. This assay is linear with respect to time and protein concentration (Gan, 1981). The main disadvantage is the limitation of the number of samples which can be analysed at one time, due to the capacity of the chromatography equipment.

As a result of the disadvantages of currently available methods described above, a new sensitive assay for the measurement of TP activity was developed which allows the processing of multiple samples simultaneously. This method exploits the ability of TP to convert 5-FU to 5-FdUrd in the presence of dR-1-P, which is more appropriate for the purposes of the current study than the conversion of thymine to thymidine. It was based on a combination of two methods. Firstly, Peters (1986) describes methods for measuring the activity of enzymes capable of catalysing 5-FU metabolism. Enzyme activities were measured in extracts prepared from freshly isolated cells or from cell pellets frozen at -70°C and enzymes were selected by the inclusion of the relevant co-substrate into the assay mixture. Substrates were then separated from products by TLC. The second method is a solid-phase extraction (SPE) method used for the purification of 5-FU and it's anabolites, followed by HPLC analysis. This method was originally developed by R. Blackie, Department of Medical Oncology, University of Glasgow for the purification and quantitation of 5-FU and 5-FdUrd from human plasma for pharmacokinetic analysis.

A new assay was designed whereby cell extracts were incubated with 5-FU in the presence of the co-substrate dR-1-P. Samples were processed via the solid-phase extraction method (to remove interfering substances present in cell extracts) and substrate and product were detected using the HPLC method described by R. Blackie.

The following chapter describes the enzyme assay method in detail and the validation steps taken to ensure optimal conditions of TP activity evaluation. Linearity

with time, protein and substrate concentration was ensured when measuring TP activity in cell lines with both high and low enzyme activities. The assay was subsequently used to measure TP activity in HT-29(TP) cells and other colon tumour cell lines. TP activity in colon cell lines was then compared with cell lines derived from other tumour types. Finally TP activity in cell lines was compared with that of human colon mucosal tissue and colon tumours.

5.2 Materials and Methods

5.2.1 Chemicals and reagents

Diaminoethanetetra-acetic acid disodium salt (EDTA), isopropanol, acetone. methanol, potassium hydroxide were purchased from Fisons (Loughborough, UK). 5-FU, Iodouracil, 5-FdUrd and bovine albumin were purchased from SIGMA (Poole, UK). Deoxyribose-1-phosphate (dR-1-P) was obtained from Fluka Chemicals (Dorset, UK). Potassium dihydrogen orthophosphate (KH_2PO_4), tetrabutylammonium phosphate and sucrose were obtained from BDH laboratory supplies (Poole, UK). Bio-Rad protein dye was purchased from Bio-Rad Laboratories GmbH (Hertfordshire, UK) Isolute NH_2 (aminopropyl) cartridges were purchased from International Sorbent Technology Ltd. (Mid Glamorgan, UK).

5.3 Cell Lines

The following cell lines were chosen to reflect a broad range of tumour types within which to assess the activity of TP.

CELL LINE	CHARACTERSITICS	SOURCE	REFERENCE
MCF-7	Metastatic breast carcinoma	ATCC (HTB 22)	Soule, 1973
ZR-75	Breast carcinoma (malignant ascitic effusion)	ATCC (CRL-1500)	Engel, 1978
WIL	NSCLC (Bronchial)	Ludwig Institute, Sutton, UK	Sri-Pathmanathan, 1994
A549	NSCLC	ATCC (CCL 185)	Giard, 1973
H125	NSCLC	NCI, USA	Gazdar, 1980 Carney, 1985
LDAN	NSCLC	Jane Plumb, University of Glasgow	Not published
CALU-1	NSCLC (Metastasis to pleura)	ATCC (HTB-54)	Fogh, 1975
SKMES	NSCLC (Pleural effusion)	ATCC (HTB-73)	Fogh, 1975
OVCAR	Ovarian Adenocarcinoma	ATCC HTB 161	Hamilton, 1983
OVIP	Ovarian Adenocarcinoma	Jane Plumb University of Glasgow	Bernard, 1985
POV	Ovarian	Jane Plumb University of Glasgow	Not published
A2780	Ovarian	Fox Chase Cancer Centre, Philadelphia	Eva, 1982
A375	Human malignant melanoma	ECACC CRL 1619	Giard, 1973
C32	Human metastatic melanoma	ATCC	Chen, 1978
HS852T	Melanoma Primary tumour	Dept. of Dermatology, Uni. Of Glasgow	Creasey, 1979
C8161	Melanoma (Abdominal wall metastasis)	Dept. of Dermatology, Uni. Of Glasgow	Welch, 1991
HS294T	Melanoma (Lymph node metastasis)	ATCC HTB 140	Creasey, 1979
G361	Malignant Melanoma	ATCC (CRL 1424)	Peebles, 1978

Table 5.1 Table outlining sources and characteristics of cell lines.

5.4 Methods

5.4.1 Preparation of cell pellets

Cells were dissociated from routine culture flasks as described in section 2.2.1.3. After dilution in 8mLs of 10% serum-containing medium the cell suspension was centrifuged at 1000g for 5 minutes at 4°C. The cell pellet was washed three times in ice-cold phosphate-buffered saline (PBS). The final pellet was snap frozen and stored at -70°C until required.

5.4.2 Preparation of cell extracts

TP activity was measured in extracts prepared from cells frozen at -70°C. Extracts were prepared by suspending the cell pellet in 200μL of 50mM Tris-HCl buffer containing 1mM EDTA (pH 7.4). The suspension was sonicated for 3 cycles of 5 seconds at maximal output with intervals of 10 seconds (during sonication the sample tube was cooled on ice). A 10,000xg supernatant (30 minutes, 4°C) was prepared and used as the source of enzyme. Protein concentration was determined by the Bio-Rad protein assay (section 2.2.3.3).

5.4.3 Enzyme assay conditions

The incubation consisted of 2.5mM dR-1-P, 0.5mM 5-FU, 2mg/mL of cell lysate and 50mM Tris-HCl, 1mM EDTA (pH 7.4). The assay was performed at 37°C in a water bath. dR-1-P, cell lysate and buffer were combined and pre-incubated at 37°C for 5 minutes and the reaction was started by the addition of the substrate, 5-FU. 50μL samples were removed at 15 minute intervals up to 1 hour and added to 200μL of isopropanol and 50μL of internal standard (Iodouracil, 50μg/mL) on ice. The samples were vortex mixed for 5 seconds. The isopropanol samples were centrifuged at 550g for 15 minutes at 4°C, the supernatants transferred to glass centrifuge tubes and evaporated

to dryness in a Haake-Bucchler vortex evaporator for approximately 20 minutes. The resultant residues were reconstituted in 100μL of 0.1M KH_2PO_4, pH 5.5 and vortex mixed twice for 10 seconds to ensure that all the material was in suspension. To this suspension was added 400μL of ice-cold acetone, followed by a vortex mix for 10 seconds. Samples were transferred to clean eppendorfs and centrifuged at 550g for 5 minutes at 4°C.

5.4.4 Solid-phase extraction method

Isolute NH_2 (aminopropyl) cartridges were conditioned with 1 mL of acetone and the supernatants were loaded onto the cartridges and the eluent collected. This was followed by a wash step with 2 mLs of methanol. Eluates were evaporated to dryness in the Haake-Bucchler vortex evaporator for 25-30 minutes. The final residue was reconstituted in 200μL of mobile-phase and 150 μL were injected onto the HPLC column, section 5.4.5.

5.4.5 HPLC method

The HPLC system consisted of a Waters WISP auto-sampler, Waters 600E pump, Waters 600E controller and Waters Photodiode array detector. Two 5mM Spherisorb (ODS-1) HPLC columns were used in series (150 X 4.6 mm followed by 250 X 4.6 mm (Crawford Scientific/Jones Chromatography).

The mobile-phase consisted of 0.02M KH_2PO_4 and 10^{-4}M tetrabutylammonium phosphate, pH 5.5 with 2M potassium hydroxide (KOH). The mobile-phase was pumped at a flow-rate of 1mL/minute and the detection wavelength was 270nm. The run-time was 30 minutes and the approximate retention times of the compounds of interest are as follows: 5-FU 8 minutes, 5-FdUrd 16 minutes and 5-IU 19 minutes (internal standard) Figure 5.1.

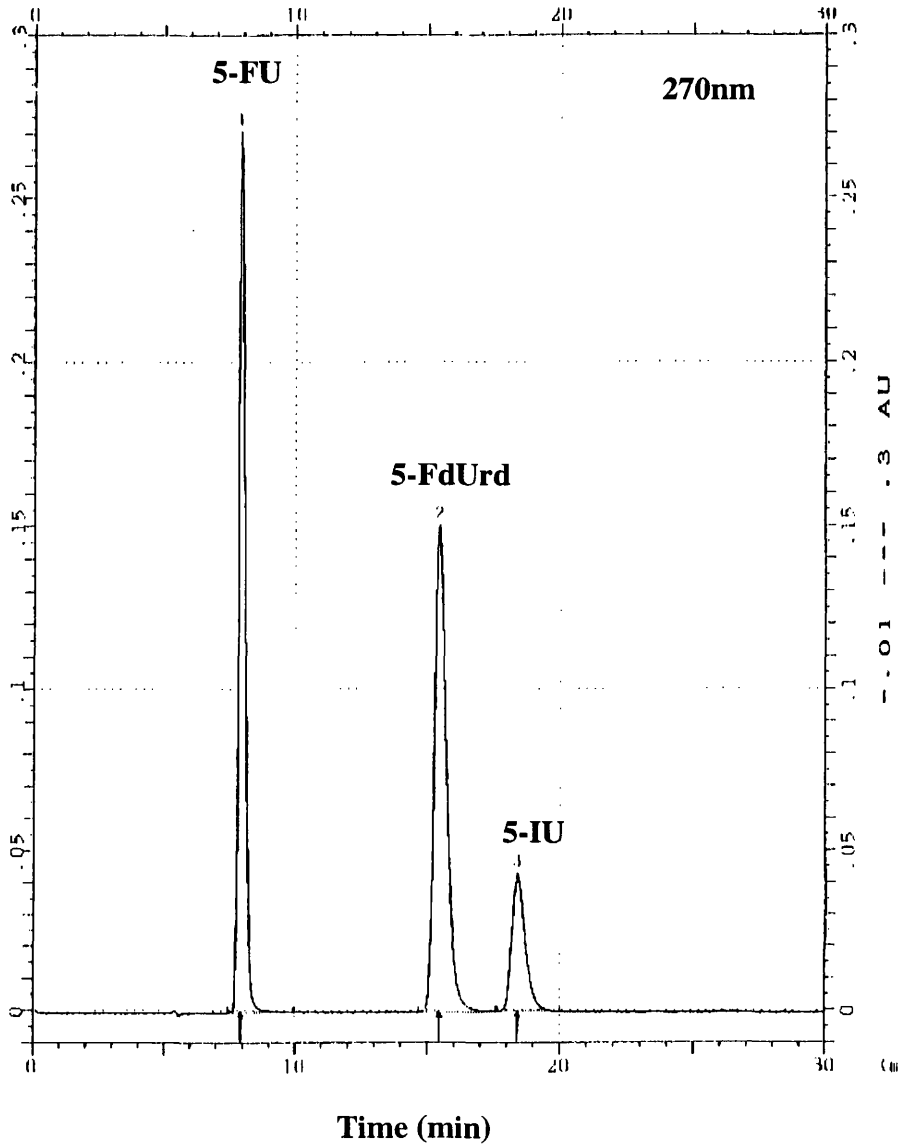


Figure 5.1 Representative HPLC chromatogram showing 5-Fluorouracil (5-FU), 5-Fluorodeoxyuridine (5-FdUrd) and 5-Iodouracil (5-IU), retention times 8, 16 and 19 minutes respectively.

5.4.6 Method Validation

5.4.6.1 Standard Curves

A range of standard samples of the analytes 5-FU and 5FdUrd were evaluated (0.05 to 10μg/mL) to define the standard curves. Analytes were serially diluted in mobile phase and 50μL injected onto the HPLC column. A plot of peak area versus concentration was created.

5.4.6.2 Accuracy

The accuracy and precision with which known concentrations of analyte could be determined was demonstrated by extraction of replicate sets of analyte samples of known concentration ranging from 0.156 to 5μg/mL. The analytes were combined with 2mg/mL of cellular protein and Iodouracil (50μg/mL). Replicates were extracted using the procedure outlined in section 5.4.4 and analysed by HPLC, section 5.4.5. Efficiencies were calculated as the percentage of the peak area of unextracted analyte. Where internal standard was included in the calculation, the ratio of the peak area of unextracted to extracted internal standard from each sample was used to correct for 5-FU and 5-FdUrd in each sample.

5.4.6.3 Co-efficients of Variation

Within- and between-day accuracy was calculated by analysis of TP activity in replicate LOVO cell pellets. Three pellets were analysed on the same day and 3 on separate days. Incubations containing dR-1-P (2.5mM), 5-FU (0.5mM), cell lysate (2mg/mL) and Tris-HCl (50mM), EDTA (1mM), pH 7.4 were assayed as described in sections 5.4.2-5.4.5.

5.4.6.4 Linearity with cellular protein concentration

Fixed concentrations of substrate (0.25mM) and co-substrate (25mM) were incubated at 37°C with a range of cellular protein concentrations (0.5-5mg/mL). Both LOVO and HT-29 cells were evaluated since they represent high and low TP enzyme expression respectively, Figure 2.2. As before, 50μL samples were removed at 15-minute intervals up to 1 hour and processed as described in section 5.4.4-5.4.5. A graph was plotted of nmoles FdUrd/min versus cellular protein concentration.

5.4.6.5 Linearity with substrate concentration

Fixed concentrations of cellular protein (2mg/mL) and co-substrate (2.5mM) were incubated at 37°C with increasing concentrations of 5-FU (0.13-1mM). As before, 50μL samples were removed at 15-minute intervals up to 1 hour and processed as described in section 5.4.4-5.4.5. A graph was plotted of nmoles FdUrd/min versus substrate concentration. HT-29 and LOVO cells were evaluated.

5.4.6.6 Linearity with time

A fixed concentration of cellular protein (2mg/mL), co-substrate (2.5mM) and substrate (0.25mM) were incubated at 37°C. As before, 50μL samples were removed at 15 minute intervals up to 1 hour and processed as described in 5.4.4-5.4.5. A graph was plotted of nmoles FdUrd/min versus time. HT-29 and LOVO cells were evaluated.

5.4.7 TP Activity in HT-29 cells transfected with TP cDNA and LOVO cells

HT-29, HT-29(V), HT-29(TP) and LOVO cells were evaluated for TP activity. Cell pellets were processed as described in section 5.4.2 and TP activity was measured under conditions of dR-1-P (2.5mM), 5-FU (0.5mM) and cellular protein (2mg/mL) over a time period of 60 minutes (section 5.4.3). Samples were processed as outlined in sections 5.4.4-5.4.5. Activity measurements were carried out in triplicate.

5.4.8 TP activity in colon, breast, lung, ovarian and melanoma tumour cell lines.

The cell lines outlined in Table 5.1 were evaluated for TP activity as above. Activity measurements were carried out in triplicate with individual cell pellets (sections 5.4.3-5.4.5).

5.4.9 5-FU cytotoxicity in Lung, Breast and Ovarian Cell Lines

5-FU cytotoxicity was measured in lung cell lines including WIL, A549, H125, LDAN and SKMES, the breast cell line MCF-7 and the ovarian cell line A2780. Cytotoxicity was quantified using the growth inhibition (MTT) assay as described in section 2.2.4.2. with a 24 hour drug exposure time. The relationship between 5-FU IC_{50} values and TP activity was investigated.

5.4.10 TP activity in colon normal/tumour biopsy tissue

Nine pairs of human normal colon and tumour tissues were evaluated for TP activity. Tissues were homogenised in 3mLs of 0.05M Tris/HCl, 0.25M sucrose, pH 7.5. Cytosolic fractions were prepared by centrifugation of tissue homogenates at 100,000g for 1 hour at 4°C. Supernatants were aliquoted and frozen at -70°C until required. Protein concentration was determined using the Bio-Rad assay outlined in section 2.2.3.3. TP activity was measured under conditions of 5-FU (0.25mM), dR-1-P (2.5mM) and cellular protein (0.25mg/mL). Initial evaluation of tissue samples using 2mg/mL protein concentration resulted in complete loss of substrate by 15 minutes and therefore the protein concentration was adjusted to 0.25mg/mL and the time course reduced to 28 minutes with 7 minute interval sampling. Samples were processed as described in section 5.4.4-5.4.5.

5.5 Results

5.5.1 Standard Curves

The standard curves for 5-FU and 5-FdUrd are shown in Figure 5.2. These curves represent the mean and standard deviation of three separate sets of data. Linearity was achieved between 0.05 and 10μg/mL with a limit of detection of 50ng/mL for each agent. These values lie well within the parameters of the assay with the range evaluated being equivalent to 0.0025-0.5mM for 5-FU and 0.0013-0.27mM for 5-FdUrd. Figure 5.3 illustrates representative HPLC chromatograms of low (50ng/mL) and high (5μg/mL) concentrations for both agents with 50ng/mL being the limit of detection of the assay for both agents.

5.5.2 Accuracy

The accuracy with which the extraction procedure (section 5.4.4) purifies 5-FU and 5-FdUrd from cell lysate incubation mixtures is shown in Table 5.2. The extraction efficiencies for 5-FU are higher than 5-FdUrd at most concentrations evaluated. As can be seen efficiency is poorer at concentrations above 2.5mM. This may be due to saturation of the extraction columns used to purify the drug from the incubation mixture (section 5.4.4). This was resolved by the inclusion of the internal standard. Correction for the internal standard resulted in extraction efficiencies greater than 77% at all concentrations analysed (Figure 5.4). Mean extraction efficiencies are 71.5 and 60% for 5-FU and 5-FdUrd respectively and 95.8 and 86.6 respectively when the internal standard is included.

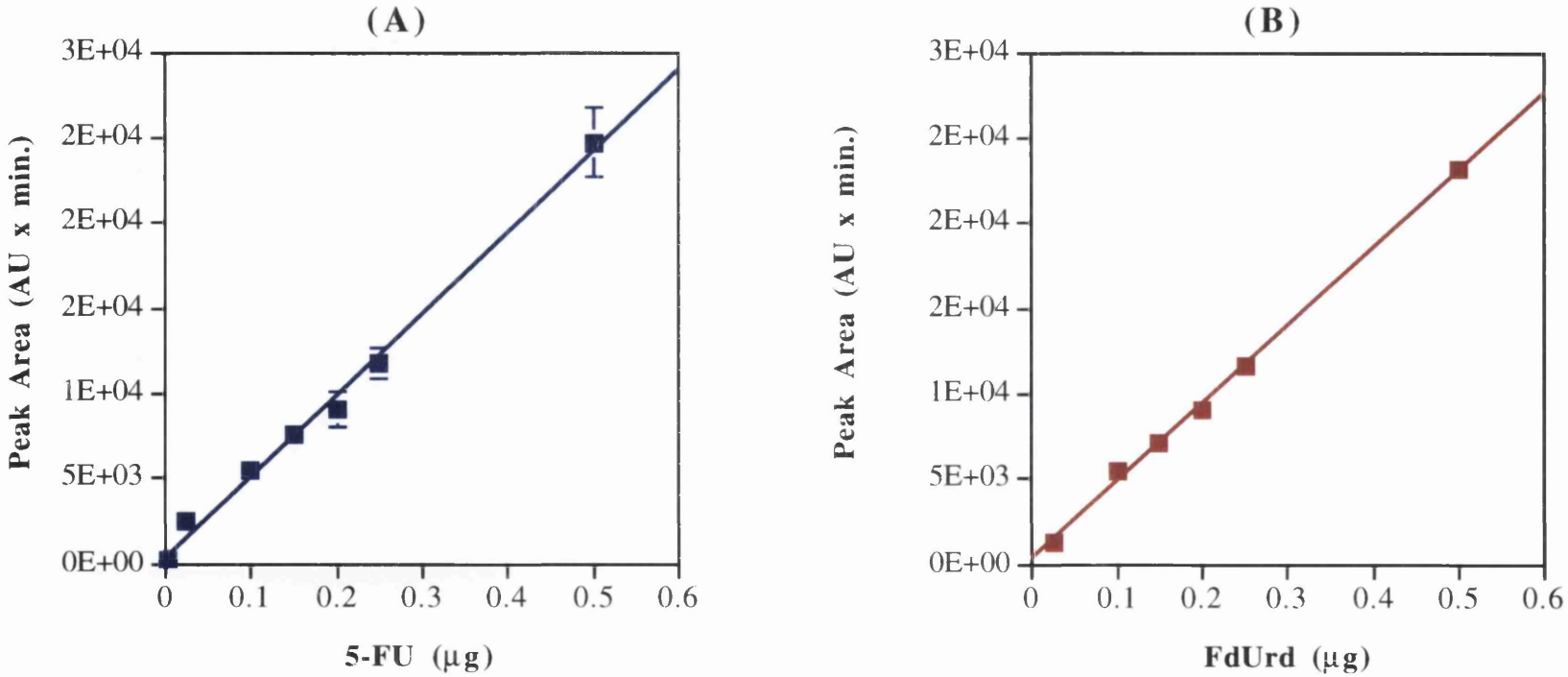


Figure 5.2 Standard curves for 5-FU, (A) and 5-FdUrd, (B). A range of standard samples of 5-FU and 5-FdUrd were evaluated, (0.05 to 10µg/mL). Analytes were diluted in mobile phase and 50µL injected onto HPLC column as described in section 5.4.6.1 and the peak area of each concentration was measured. Correlation co-efficient is 0.99 for 5-FU and 5-FdUrd.

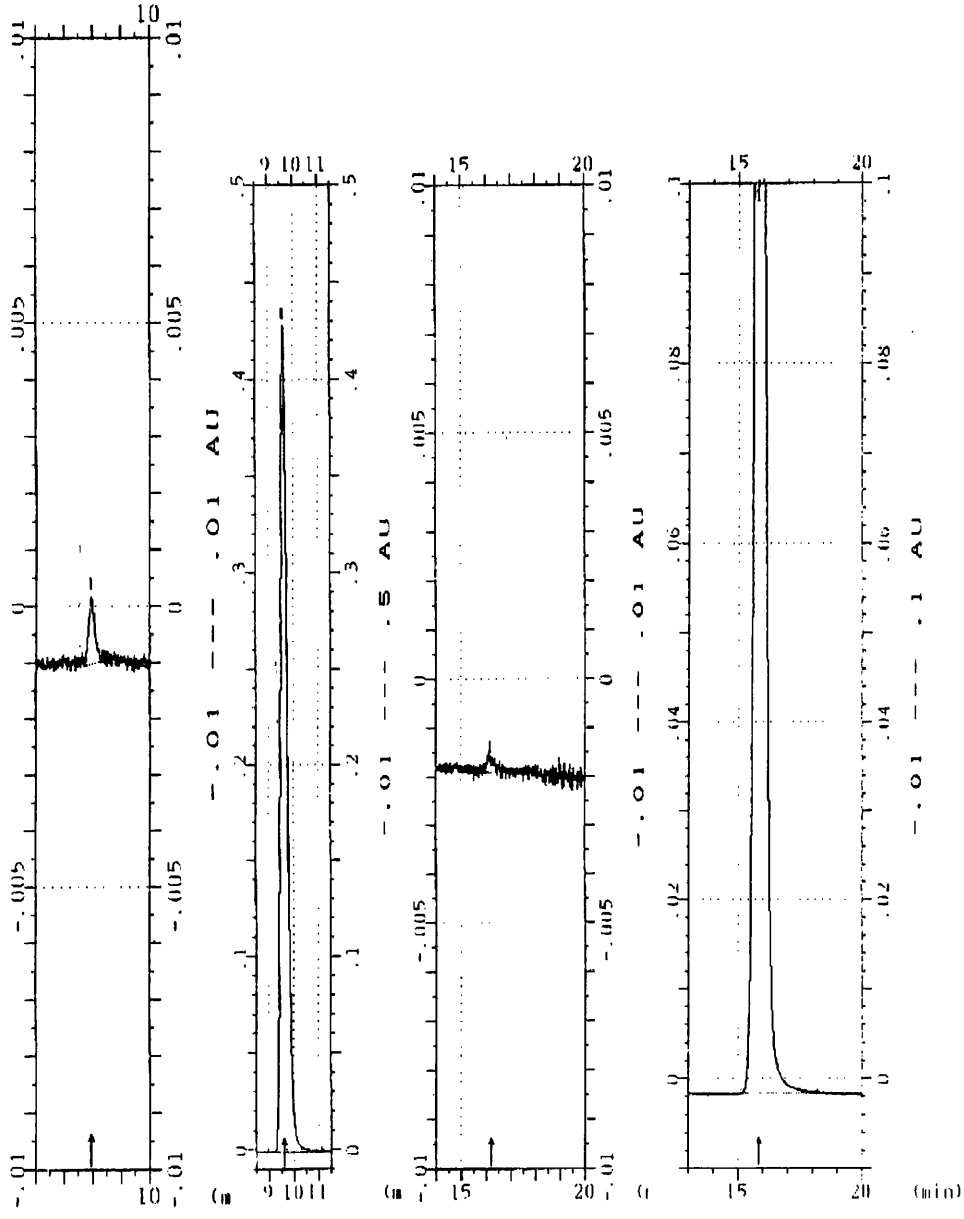


Figure 5.3 Representative chromatograms of 5-FU and 5-FdUrd at high and low concentrations. 5-FU and 5-FdUrd were serially diluted in HPLC mobile phase to a range of concentrations and 150µls was injected onto the HPLC column under the conditions described in section 5.4.5. Chromatograms representing 50ng/ml and 5µg/ml for each drug are illustrated.

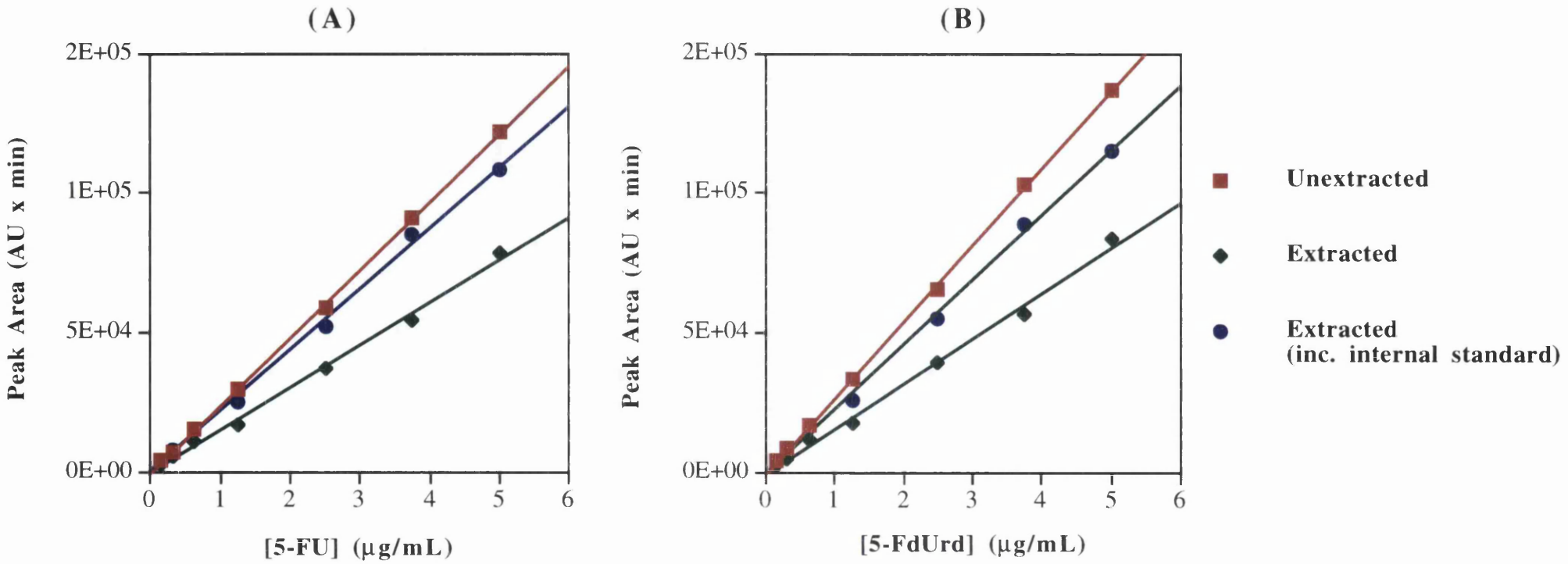


Figure 5.4 Efficiency of extraction procedure (outlined in section 5.4.6.2) for 5-FU, (A) and 5-FdUrd, (B). Replicate samples of known concentrations of both analytes (ranging from 0.156 to 5µg/mL) were co-extracted along with 2mg/mL of cellular protein and 50µg/mL of Iodouracil (internal standard). Efficiency of extraction with and without correction by internal standard (iodouracil) is illustrated. Percentage extraction efficincies are outlined in Table 5.2.

[Drug] (mM)	% Extraction Efficiency			
	5-FU		5-FdUrd	
	- Internal Standard	+	- Internal Standard	+
0.156	84.9±18.2	116.2±5.4	53.2±10.7	91.1±6.6
0.3125	77.2±3.2	108.2±13.1	60.5±4.1	88.5±5.1
0.625	71.1±6.7	92.4±3.4	58.2±1.7	95.6±4.4
1.25	72.9±7.2	85.6±0	67.8±7.6	77.4±0
2.5	71.9±0	85.6±0	68.2±0	84.1±15.2
3.75	62.9±12.7	93.7±9.7	59.5±12.2	85.8±8.7
5.0	59.5±13.2	89.2±0	54.5±12.0	83.9±0
Mean Extraction Efficiency	**71.5±7.8**	**95.8±10.9**	**60.3±5.4**	**86.6±5.4**

Table 5.2 Extraction efficiencies for a range of concentrations of 5-FU and 5FdUrd when combined with cellular protein. Efficiencies were calculated with and without inclusion of the internal standard, Iodouracil. Values represent means of replicate samples.

5.5.3 Co-efficients of Variation

The intra- and inter-assay co-efficients of variation for the measurement of TP activity in LOVO cells were 10.98% and 23.6% respectively. These measurements were taken over 3 days.

5.5.4 Linearity with Cellular Protein Concentration

Increasing concentrations of cellular protein were incubated with a fixed concentration of substrate and co-factor and FdUrd formation was quantified by HPLC. TP activity increased with protein concentration in a linear fashion in cell lines with low and high enzyme activities (r=0.96 and 0.99 for HT-29 and LOVO cells respectively), Figure 5.5. Values are means and standard deviations of three individual experiments.

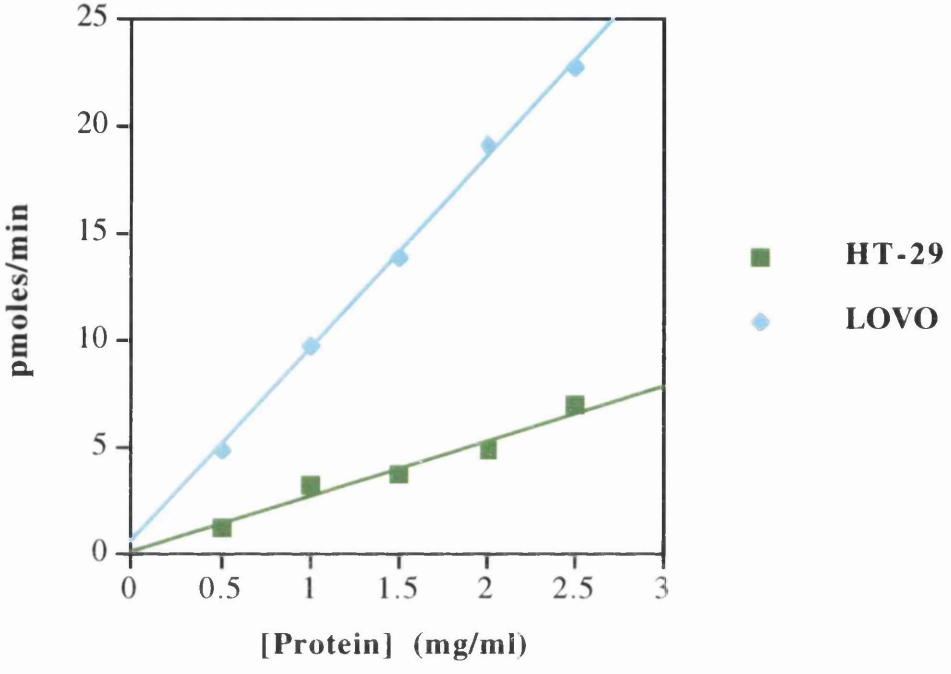


Figure 5.5 Linearity of TP activity with cellular protein concentration in HT-29 and LOVO cells. Fixed concentrations of 5-FU (0.5mM) and co-substrate (2.5mM) were incubated at 37°C with increasing concentrations of cellular protein, samples were removed at 15 minute intervals, extracted and injected onto the HPLC column as described in section 5.4.6.4. pmoles of FdUrd produced per minute was plotted against protein concentration. TP activity was linear with respect to protein concentration up to 2.5mg/ml in HT-29 and LOVO cell lines, (correlation co-efficients 0.96 and 0.99 respectively).

5.5.5 Linearity with Substrate Concentration

Increasing concentrations of substrate were incubated with a fixed concentration of cellular protein and co-factor. LOVO cells were evaluated and TP activity was shown to increase with substrate concentration in a linear fashion in cells from 0.13-0.25mM however concentrations higher than this appeared to result in saturation of the enzyme Figure 5.6. Values are means and standard deviations of three independent experiments.

5.5.6 Linearity with Time

A fixed concentration of substrate (0.5mM), cellular protein (2mg/mL) and co-factor (2.5mM) were incubated at 37°C and TP activity was measured over one hour in HT-29 and LOVO cells. As can be seen in Figure 5.7 the formation of 5-FdUrd is linear with respect to time over one hour with a correlation co-efficient (r) of 0.99 for both LOVO and HT-29. An incubation time of greater than one hour was not evaluated. Since TP activity can be measured in HT-29 (with the lowest measurable activity) over this time period a longer incubation would only prolong the time taken to complete the assay.

5.5.7 TP Activity in Cell Lines

5.5.7.1 TP activity in Transfected HT-29 cells

Following validation of the enzyme activity assay, TP activity was measured in HT-29(TP), HT-29(V) and the parental cells. Activity was also measured in LOVO cells. As can be seen in Figure 5.8, TP activity was unchanged in HT-29(V) cells compared with HT-29 parental cells with activity of 2.0 pmoles FdUrd formed per minute per mg of cellular protein (pmoles/min/mg). HT-29(TP) had approximately 5-fold higher TP activity (10.0 pmoles/min/mg) than the controls and LOVO cells had approximately a further 2.5-fold increase in activity (25.0 pmoles/min/mg).

5.5.7.2 TP Activity in a Range of Tumour Cell lines

TP activity was measured in a range of tumour cell lines including colon (HT-29, DLD-1, CACO-2, BE and LOVO) breast,(MCF-7, ZR-75) lung,(WIL, A549, H125, LDAN, CALU and SKMES) ovarian (OVCAR, OVIP, POV and A2780) and melanoma cells (A375, C32, HS853T, C8161, HS294 and G361), see Table 5.3. As seen in Figure 5.9, within each tumour type (with the exception of ovarian) there is a wide range in TP activity with the majority of cell lines having low activity.

5.5.8 5-FU Cytotoxicity in Cell Lines

5-FU cytotoxicity was investigated in a number of cell lines from various tumour sources using the MTT assay and representative dose-response curves are illustrated in Figure 5.10. The respective 5-FU IC_{50} values are outlined in Table 5.3. There was approximately a 70-fold range in IC_{50} values obtained, ranging from 1.1 to 75.2μM.

The relationship between 5-FU cytotoxicity and TP activity was investigated by plotting 5-FU IC_{50} versus TP activity. There appeared to be no relationship between these two parameters (r=1.28) as shown in Figure 5.11. Two groups of cell lines emerge in Figure 5.11. One group with high TP activity the other with low activity, however within these two groups there is heterogeneity within the range of 5-FU IC_{50} values.

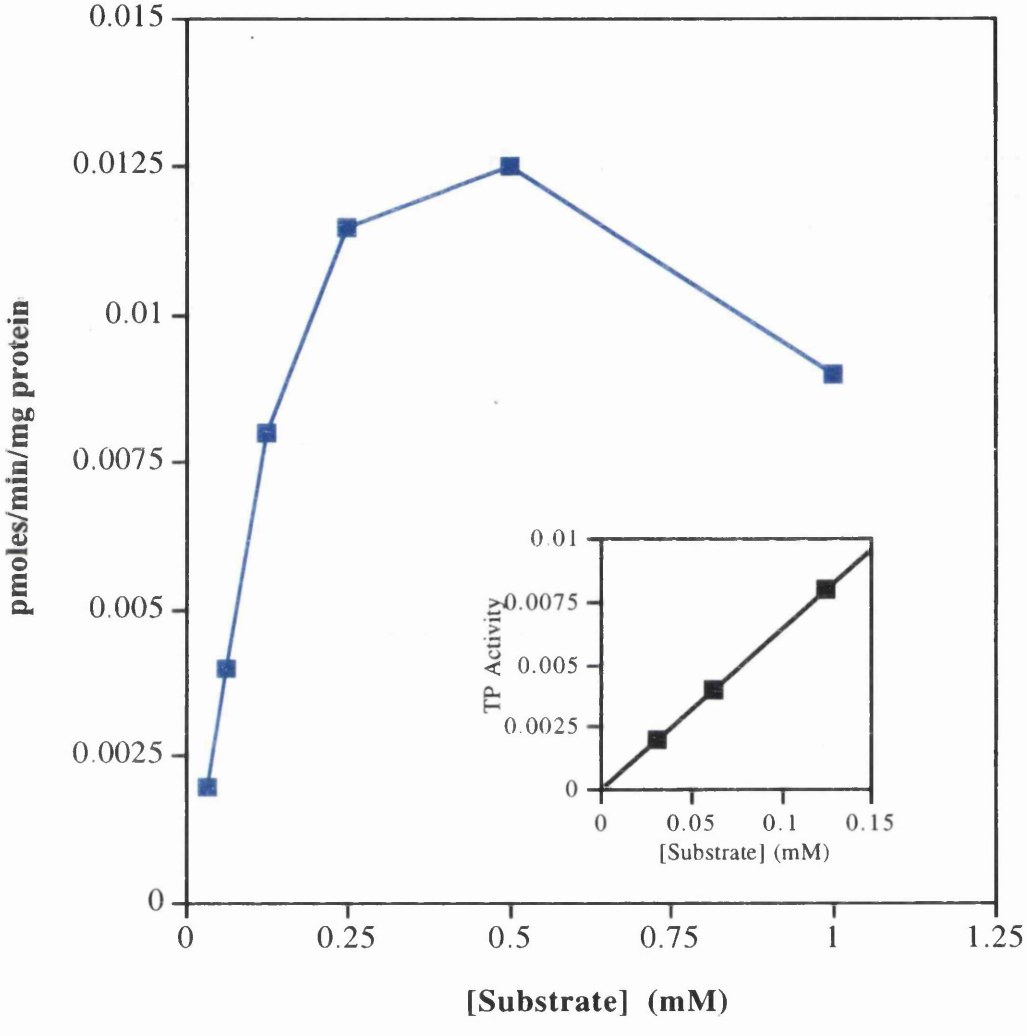


Figure 5.6 Linearity of TP activity with substrate concentration in LOVO cells. Fixed concentrations of cellular protein (2mg/ml) and co-substrate (2.5mM) were incubated with increasing concentrations of 5-FU. Samples were removed at 15 minute intervals, extracted and injected onto the HPLC column as described in sections 5.4.3-5.4.5. TP activity was linear with substrate concentration up to 0.125mM (see inset plot, linear regression analysis, TP Activity = 0.06 (Substrate concentration), r=1.0)

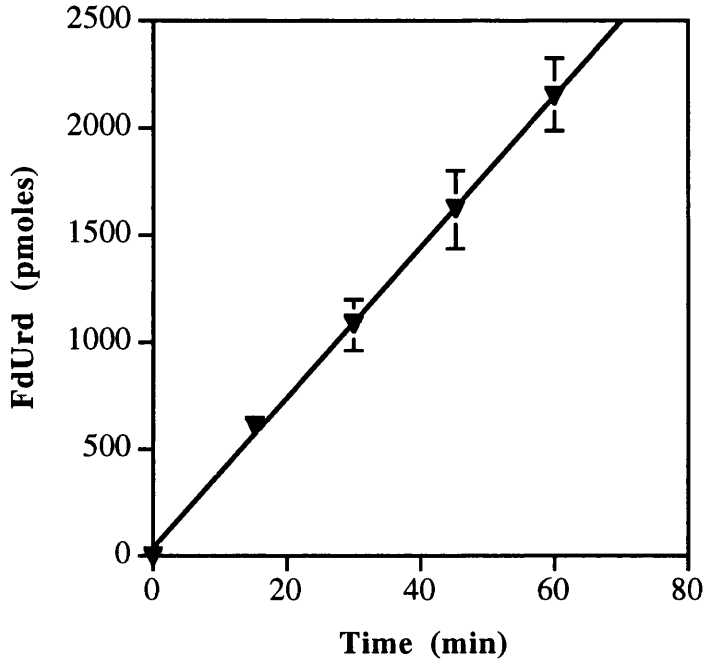


Figure 5.7 Linearity with time. Graph of FdUrd formation with respect to time in LOVO cells (r=1.0). 2mg/mL of LOVO cellular protein, 2.5mM dR-1-P, 0.5mM 5-FU were incubated at 37°C. Samples were removed at 15 minute intervals and 5-FdUrd product quantitated. Measurements were carried out in triplicate.

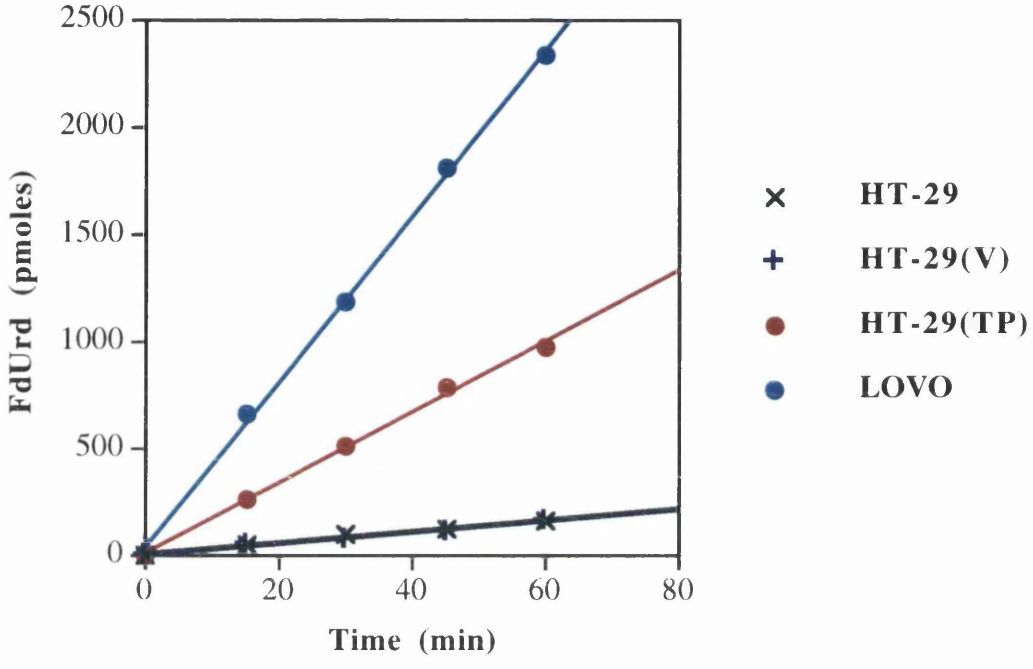


Figure 5.8 TP activity in HT-29 cells transfected with TP compared with the parental cells, HT-29, the vector alone controls, HT-29(V) and LOVO cells as a positive control. 2mg/mL of cellular protein was incubated with 2.5mM dR-1-P and 0.5mM 5-FU at 37°C. Samples were removed at 15 minute intervals and 5-FdUrd poduct was quantified by HPLC. Values are representative of a single experiment. TP activity was 2.0, 2.0, 10.2, and 25.0 pmoles/min/mg of protein respectively.

CELL LINE	TP ACTIVITY (pmoles/min/mg protein)
Colon	
HT-29	2.0
HT-29(V)	2.0
DLD-1	2.0
CACO-2	2.7±1.2
BE	3.4±1.2
HT-29(TP)	10.2±3.5
LOVO	25.0±2.4
Breast	
MCF-7	7.4±3.8
ZR-75	24.9±9.3
Lung	
WIL	0
A549	4.1±0
H125	4.6±0.8
LDAN	4.7±3.1
CALU	7.4±1.2
SKMES	25.7±6.5
Ovary	
OVCAR	0
OVIP	1.1±0.7
POV574	44.6±6.7
A2780	3.25±2.1
Melanoma	
A375	0.7±0.2
C32	3.3±1.3
HS852T	3.6±0.1
C8161	4.5±1.3
G361	30.7±8.3

Table 5.3 TP activity in colon, breast, lung, ovarian and melanoma cell lines. Enzyme activity was measured in cell lysate preparations as described in sections 5.4.3-5.4.5.

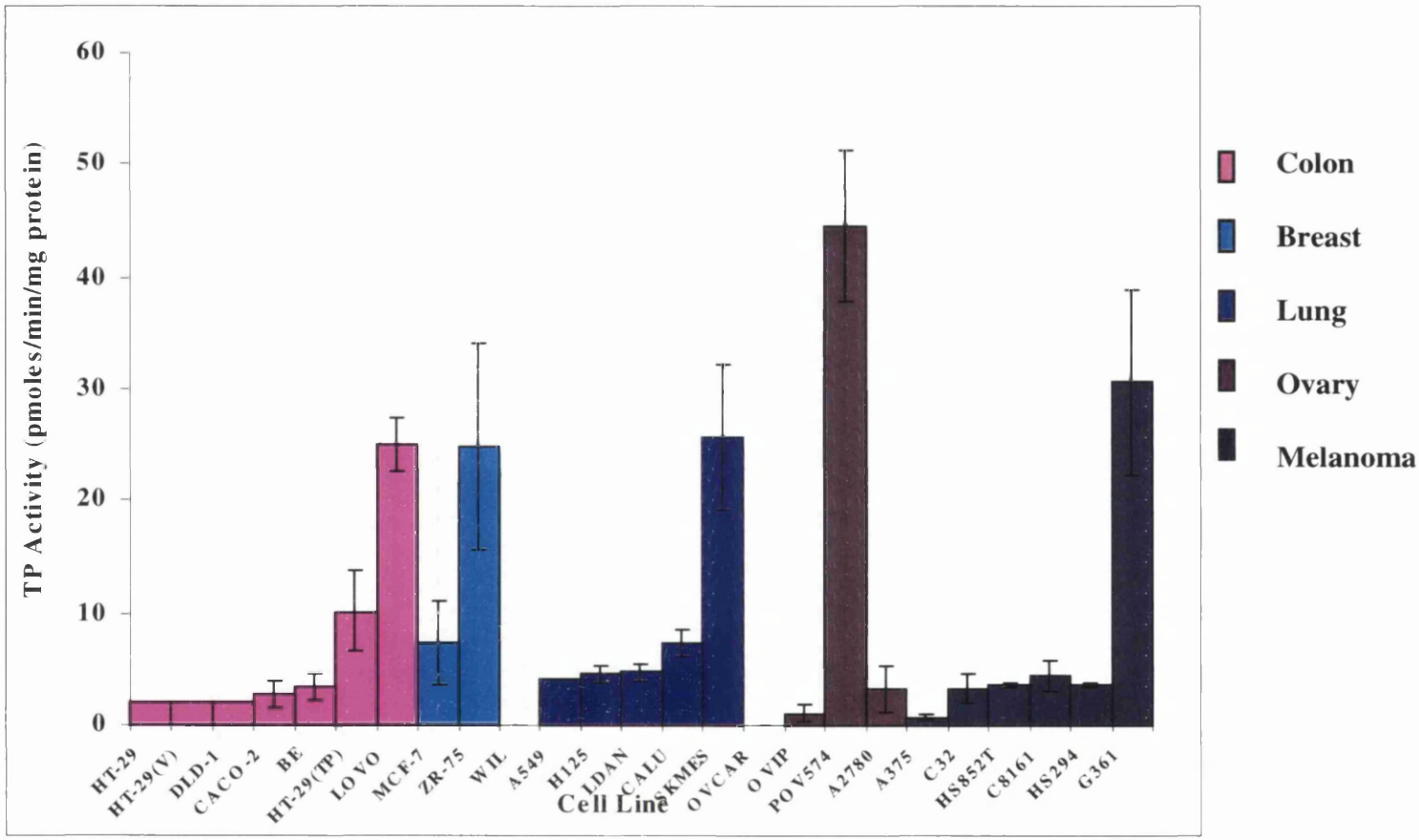


Figure 5.9 Histogram of TP activity in cell lines derived from various tumour types; colon, breast, lung, ovary and melanoma. Enzyme activity was quantitated as described in section 5.4. Values represent means and standard deviations of triplicate independent measurements.

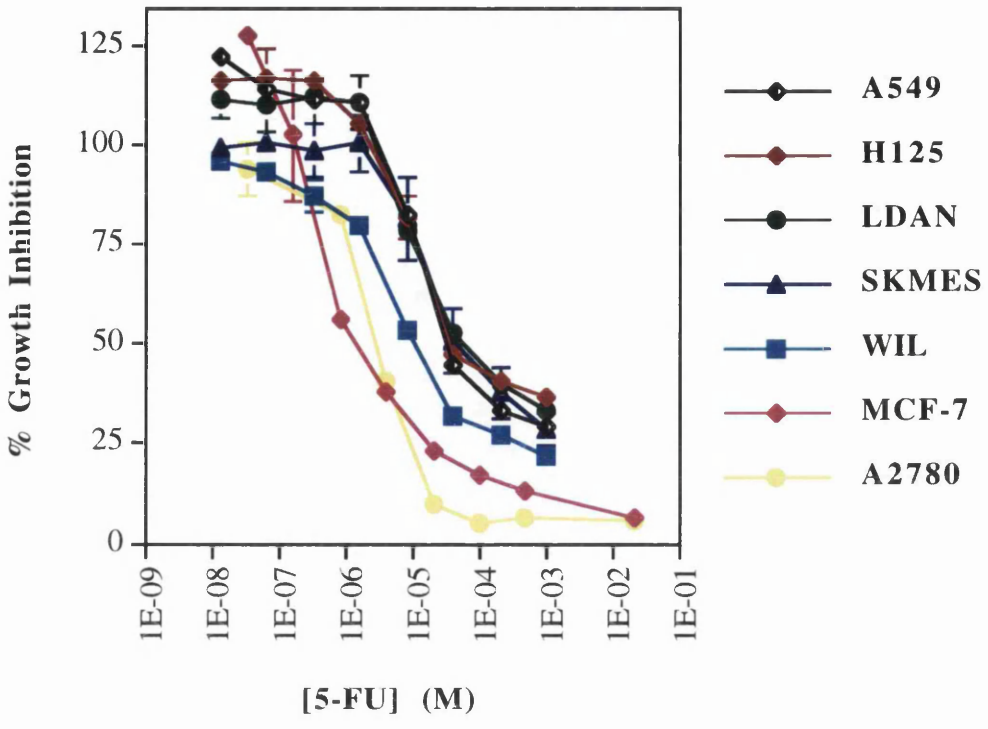


Figure 5.10 Dose response curves of 5-FU cytotoxicity in a range of tumour cell lines. Cell lines included five lung lines (A549, H125, LDAN, SKMES and WIL) and one breast, (MCF-7) and one ovarian cell line, (A2780). Cytotoxicity was measured by the MTT assay as described in section 2.2.4.2. Values are means ± standard errors of three plates, 8 wells per plate. 5-FU IC_{50}'s are outlined in Table 5.3.

Cell Line	A549	H125	LDAN	SKMES	WIL	MCF-7	A2780
5-FU IC_{50}	31.3 ±3.3	38.9 ±11.4	48.8 ±20.2	75.2 ±11.3	10.1 ±1.0	1.1 ±0.72	3.16 ±0.60

Table 5.3 Table of 5-FU IC_{50} values in tumour cell lines. 5-FU cytotoxicity was measured using the MTT assay and dose response curves are outlined in Figure 5.10. Values are means and standard deviations of 3 plates.

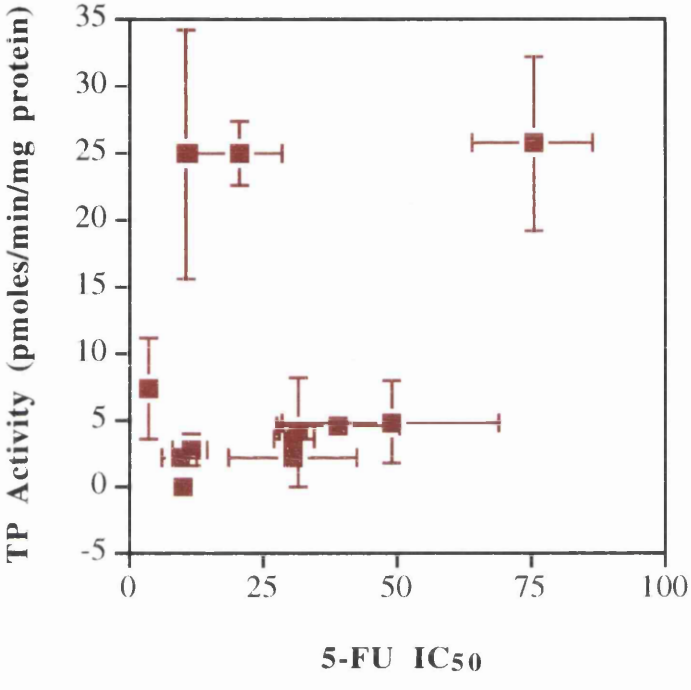


Figure 5.11 Graph of TP enzyme activity (nmoles/min/mg protein) versus 5-FU IC_{50}. 12 cell lines were evaluated for a relationship between TP enzyme activity and sensitivity to 5-FU; HT-29, DLD-1, CACO-2, BE, LOVO (colon), MCF-7 (breast), A2780 (ovarian), SKMES, H125, LDAN, WIL, and A549 (lung). TP activity was measured as described in section 5.4 and 5-FU cytotoxicity was determined using the growth inhibition (MTT) assay (section 2.2.4.2). Values are means and standard deviations of three determinations of both 5-FU IC_{50} and TP activity.

5.5.9 TP Activity in Colon Tumour/Normal Biopsy pairs

TP activity was also measured in nine pairs of human colon tumour and normal biopsy pairs. In seven out of the nine pairs tested, TP activity was greater in the tumour compared to the normal tissue, Figure 5.12. In one pair (no. 7) TP activity was much greater in the normal than the tumour. These tissues are from a different source than those analysed in chapter 3, Figure 3.2, however the results are very similar. Details concerning the tumour location and stage of disease are outlined in the table below.

Patient No.	TP Activity (nmoles/min/mg protein) Normal/Tumour		Tumour site	Size	State of Differentiation	Duke's Stage
1	243.7	206.3	colon	5x4	moderate	B
2	229.1	757.1	caecum	7x5	moderate	C
3	130.0	783.1	caecum	11x7	well	B
4	207.9	800.9	caecum	7x5	moderate	B
5	316	713.2	sigmoid colon	5x1.5	well	B
6	440	809.1	sigmoid colon	2x2	moderate	A
7	804.2	259.1	ascending colon	4x3	moderate	A
8	229.9	395.6	rectum	8x8	moderate	C
9	192.5	260.8	sigmoid colon	4x4	moderate	D

Table 5.4 Patient details for each of the colon tumour/normal biopsy pairs analysed. Samples were obtained from Dr Howard L. McLeod of the Department of Medicine and Therapeutics at the University of Aberdeen. TP activity was measured in both normal colon and tumour tissue and the results are illustrated in Figure 5.12.

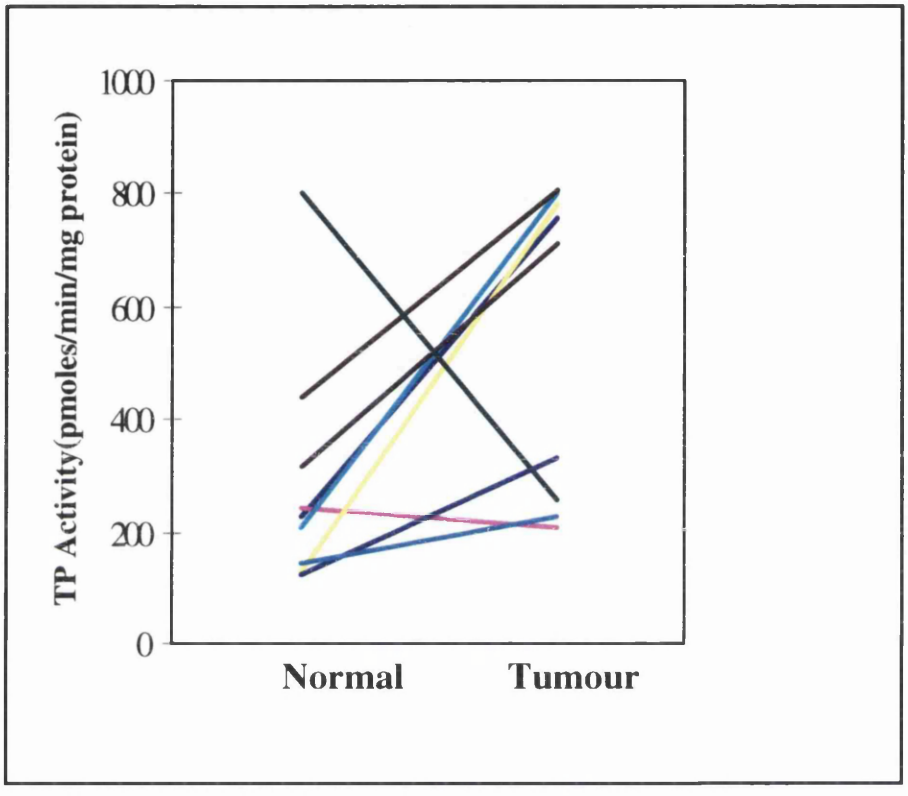


Figure 5.12 Graph of relative TP activity (pmoles/min/mg protein) in normal and tumour tissue from 9 biopsy pairs, each represented by a single line. Activity measurements were made as described in section 5.4, with cellular protein (0.25mg/mL), 5-FU (0.5mM), dR-1-P (2.5mM) and 50mM tris-HCl, 1mM EDTA. The time course of the assay was reduced to 28 minutes, (normally 60 minutes for cell line activity measurements). Values are from a single representative experiments. Satisfactory repeat measurements were made.

5.6 Discussion

A novel assay was developed for the measurement of TP enzyme activity in tumour cell lines. The main purpose of the assay was to determine whether the increase in TP protein expression observed in HT-29(TP) cells (Figure 4.3) reflected an increase in enzyme activity.

The present method involved assessing the ability of TP to catalyse the conversion of 5-FU to 5-FdUrd in the presence of the co-substrate, dR-1-P. Other methods used thymine and thymidine as substrates (Gan, 1981 and Freidkin and Roberts, 1953) however since 5-FU cytotoxicity is the focus of the present study it was thought to be a more appropriate substrate.

TP extracted from tumour cells, catalysed the conversion of 5-FU to 5-FdUrd in a linear fashion over one hour allowing a rate of reaction to be measured. No other metabolites such as 5-FdUMP were detected by HPLC when compared against standards. TP did not appear to catalyse the reverse reaction i.e., the phosphorolysis of 5-FdUrd to 5-FU, since there was no decrease in the amount of 5-FdUrd after its formation throughout the time course analysed. There was no 5-FdUrd formed when dR-1-P was absent from the reaction. There was also no spontaneous conversion of 5-FU to 5-FdUrd when the enzyme source was absent. Conversion of 5-FU to 5-FdUrd was dependent upon enzyme and co-factor.

The activity assay was linear with respect to cellular protein concentration, substrate concentration and time when measuring activity in cell lines with both low (HT-29) and high (LOVO) activity. Parental HT-29 cells have the lowest measurable TP activity.

The intra- and inter-assay variability was 10.98% and 23.6% respectively. The summary report of the conference on "Analytical Methods Validation: Bioavailability,

Bioequivalence and Pharmacokinetic Studies" in 1992 stated that the co-efficient of variation should not exceed 15% (Shah, 1992). Using this as a guideline, it would therefore appear that the intra-assay variability of the assay is acceptable. The presence of the internal standard ensures low variability. Inter-assay variability was, however, higher than the normally accepted level (23%).

This assay was more sensitive than immunoblotting. TP protein could not be detected in four out of five colon cell lines evaluated (Figure 2.2) however TP activity was measured in all five cell lines. Within the wider range of cell lines TP activity was undetected in only 2 out of 24 cell lines.

A larger number of samples can be assessed using the present method compared to the TLC/radiochemical method described by Gan (1981). Up to 12 cell lines can be evaluated in one day using this method, however this does depend on the capacity of the equipment necessary to process samples, e.g. vortex evaporator capacity.

The presence of the internal standard Iodouracil proved invaluable. During each experiment it is possible to have variable solid-phase extraction efficiencies between cell lines (since only two cell lines can be extracted simultaneously) or to lose a fraction of the sample due to spillage or breakage of tubes. The inclusion of the internal standard resulted in final extraction efficiencies of greater than and 95.8 and 86.6% for 5-FU and 5-FdUrd respectively (Table 5.2).

This validated and sensitive assay confirmed that HT-29(TP) cells expressed an increase in TP protein expression, which was 5-fold greater than controls. Both HT-29(V) and HT-29 cells had similar TP activity. The 5-fold increase in activity lead to a 1.6-fold increase in 5-FU sensitivity. LOVO cells had a further 2.5-fold increase in TP activity, which was also observed by Western immunoblotting.

Having shown that transfection could increase TP activity in HT-29 cells, enzyme activity was measured in a further 19 cell lines in order to determine how HT-

29 cells compared with other colon cell lines and also cell lines derived from other tumour types. This assay was therefore used for the measurement of TP activity in 24 cell lines from various tumour types including colon, breast, ovarian, lung and melanoma tumours. Activity was heterogeneous within each group of cell lines, Figure 5.9. There was no prominent tumour type, which contained cell lines with high TP activity compared with other tumours.

TP activity does appear to reflect TP protein expression in colon cell lines. TP protein was not detected in BE, HT-29, CACO-2 and DLD-1 and these cell lines had very low TP activity. On the other hand, TP activity was high in LOVO cells in which TP protein was easily detected by Western immunoblotting. TP activity correlates with protein and mRNA expression in lung cell lines (Heldin, 1993). TP activity was also found to be heterogeneous, with a 192-fold range in activities using the spectrophotometric method.

The second question addressed was whether increased TP activity measured using this sensitive assay correlates with cytotoxicity. TP activity did not correlate with sensitivity to 5-FU in colon, lung, breast and ovarian cell lines (r=0.28). For example, in the lung cell lines, WIL has no measurable TP activity however this cell line was the most sensitive lung cell line to the effects of 5-FU. As can be seen in Figure 5.11 there are two distinct groups of cell lines; those with low TP activity and those with high TP activity. Within each group there was similar variability in 5-FU IC_{50}, thus confirming that TP activity alone does not determine sensitivity to 5-FU and gives some explanation as to why a 5-fold increase in TP activity resulted in only a 1.6-fold decrease in 5-FU IC_{50} in HT-29(TP) compared with HT-29(V)cells. Studies in patients with colorectal tumours have demonstrated that tumours with the highest TP expression were non-responders to 5-FU and LCV treatment (Metzger, 1998). Of the patients who responded, a broad range of activities was observed.

In chapter 3 TP protein expression was measured by Western immunoblotting of tumour and normal colon pairs from a different source than those used in the present study. TP protein expression was elevated in all tumour tissues compared with their normal counterparts and this is consistent with other studies as discussed in that chapter. In the present study TP activity was measured in another set of tumour and normal colon pairs and a similar pattern emerged. In seven out of nine pairs, TP activity was greater in the tumour compared with the normal tissue, with a range of 1.35- to 6.02-fold elevation. In one of the remaining pairs TP activity was unchanged. In another pair TP activity was 3.1-fold greater in the normal tissue compared with the tumour. The general trend was however the same for TP activity compared with protein expression for colon tumours, i.e, there is an elevation in tumour versus normal tissue. In the study carried out by Luccioni *et al* (1994) TP activity was also elevated in human colon tumour tissue compared with normal tissue with a mean increase in activity of 2.3-fold (ranging from 2.1 to 2.9-fold). An elevation in the activity of other enzymes involved in the pyrimidine nucleotide pathway such as thymidylate synthase, thymidine kinase, uridine kinase and uridine phosphorylase was also found (Luccioni, 1994).

This study has demonstrated that TP protein levels and TP activity are reduced in cell lines compared to tumours and therefore many of the tumours from which the cell lines were derived may have had higher TP activity which was down-regulated when cell lines were established. This brings into question therefore the suitability of colon cell lines as models of colon tumours.

5.6.1 Summary

A sensitive enzyme assay has been developed for measuring TP activity (in catalysing the formation of 5-FdUrd from 5-FU) which allows the evaluation of up to 12 cell lines in one day. This assay was used to assess TP activity in HT-29(TP) cells. TP

activity was shown to be elevated 5-fold when compared with HT-29(V) and HT-29 thus confirming the success of the transfection experiments. It was also used to measure TP activity in a range of tumour cell lines. TP activity was heterogeneous across the range of tumour types analysed. TP activity was greatly reduced in cell lines compared to tumour tissue and normal colon. As seen with TP protein expression in human colon tumour and normal biopsy pairs, section 3.3, Figure 3.2, there is a consistent pattern of elevation of TP activity in tumours and this confirms current biochemical data. Most importantly, TP activity alone did not determine the sensitivity of the cell lines to 5-FU.

CHAPTER 6

6. Exploring Potential Rate-limiting Factors in the Activation of 5-FU to 5-FdUMP

6.1 Introduction

The human colon carcinoma cell line HT-29(TP) created from HT-29 cells has increased TP expression and activity, unaltered growth kinetics and TS expression compared with the parental and vector alone control cells. The purpose of creating this cell line was to determine the role of increased TP expression on cellular sensitivity to 5-FU.

HT-29(TP) cells were 1.6-fold more sensitive to 5-FU compared to the vector alone control cells, HT-29(V) when assessed using the MTT assays. There was no difference in 5-FU cytotoxicity between HT-29(TP) and HT-29 cells. There was no notable increase in TS inhibition in HT-29(TP) cells compared with HT-29 and HT-29(V) cells when quantitated as the ratio of bound to free enzyme (Figures 4.7 and 4.8). The aim of this chapter was to investigate the potential for enhancing this small increase in sensitivity to 5-FU in the HT-29(TP) cells. There are a number of factors which contribute to 5-FU cytotoxicity, in particular in the direct anabolism of 5-FU to 5-FdUMP and subsequent TS inhibition (see Figure1.1). Modulation of these factors was evaluated using the MTT assay.

6.1.1 Deoxyribose-1-Phosphate

TP catalyses the direct deoxyribosyl transfer reaction converting thymine/5-FU to thymidine/5-FdUrd in the presence of the co-substrate deoxyribose-1-phosphate (dR-1-P). Sub-optimal intracellular concentrations of dR-1-P and deoxyribose donors (e.g. thymidine) are thought to limit the activity of TP despite it's increased expression in tumours (Breitman, 1964 and Gallo, 1967). Deoxyinosine treatment results in an

increase in TS inhibition mouse and human sarcoma cell lines (Evans, 1981). This was demonstrated *in vitro* by Schwartz and colleagues who found only a 3-fold increase in thymine incorporation in cells in which there was a 12-fold increase in TP enzyme activity (Schwartz, 1994). IFN-α treatment caused a 1.8-fold potentiation of 5-FU cytotoxicity and the addition of the deoxyribose donor 5-propynyloxy-2'-deoxyuridine (PO-Urd) resulted in an overall potentiation of 25-fold. PO-Urd increased thymine incorporation, reduced 5-FU IC_{50} and enhanced TS inhibition. The possibility, therefore, that the concentration of the co-substrate for TP, dR-1-P is rate-limiting in HT-29(TP) cells was investigated.

6.1.2 Dialysed Serum

The extent to which 5-FU is phosphorylated to 5-FdUrd depends, in part, on the concentration of intracellular pyrimidines which compete with 5-FU for the active site on TP. Foetal bovine serum, used in the medium for routine culture of cell lines, contains low levels of pyrimidines Therefore the possibility that this may be masking any increase in TP activity in HT-29(TP) cells was investigated. 5-FU cytotoxicity was therefore measured in HT-29, HT-29(V) and HT-29(TP) cells using the MTT assay with dialysed serum-containing medium throughout the assay procedure. The sequence of dialysed followed by normal serum was also investigated where dialysed serum was present in the medium when cells were seeded into 96-well plates and during treatment with 5-FU, then during the recovery period following drug treatment, normal serum-containing medium was re-introduced. This allowed investigation of the possibility that pyrimidines in the medium could reverse TS inhibition following 5-FU treatment.

6.1.3 Leucovorin

TS enzyme catalyses the methylation of dUMP to dTMP and 5,10-CH_2THF is the essential methyl donor in this reaction (Figure 1.3). High levels of reduced folates

are necessary for optimal binding between 5-FdUMP and TS. They are also required for the stabilisation of the ternary complex and this is a critical determinant of sensitivity to 5-FU.

Polyglutamated folates are more potent in stabilising TS inhibition than the monoglutamated forms. Folates with 3 or 6 glutamate residues are 18- and 200-fold more effective, respectively, in stabilisation of the ternary complex than the monoglutamated form (Radparvar, 1989; Yin, 1982; Evans, 1981, Moran and Scanlon, 1991). Prolonged exposure is, therefore, necessary to permit accumulation of the more potent polyglutamates (Houghton, 1990; Boarman, 1992).

LCV is the exogenous precursor of 5,10-CH_2THF. LCV is transported into the cell via the reduced folate carrier and subsequently metabolised to other folate intermediates which can be polyglutamated, enabling long retention of these reduced folates including 5,10-CH_2THF in the cell. LCV increases the cytotoxicity of 5-FU *in vitro* (Yin, 1982, Keyomarsi, 1986, Houghton, 1991 and Drake, 1995) in a concentration and time-dependent manner. The results of several pre-clinical studies suggest that LCV be administered prior to or concurrently with 5-FU to permit metabolism to polyglutamates (Nadal, 1988 and 1989).

The apparent limited increase in sensitivity of HT-29(TP) cells to 5-FU compared to HT-29(V) cells, may therefore be attributed to sub-optimal concentrations of reduced folates necessary for stabilisation and retention of the ternary complex formed between TS and 5-FdUMP. Cells were pre-treated with LCV (10μM) for 24 hours after which time they were treated with increasing concentrations of 5-FU. A delay between LCV and 5-FU was also investigated to allow for folate polyglutamation. A 6 hour delay was chosen as a result of TS inhibition studies in MCF-7 and NCI H630 cells (Drake, 1995). In these studies maximum TS inhibition was achieved when an interval of 4 and 18 hours was introduced between 5-FU and LCV treatments.

6.1.4 Thymidine

TS inhibition has been demonstrated in HT-29, HT-29(V) and HT-29(TP) cells by immunoblotting studies (Figures 4.7 and 4.8). Approximately 50% TS inhibition was demonstrated at 5-FU IC_{10} and IC_{50} concentrations, but its contribution to overall cytotoxicity was not been assessed. The question of whether TS inhibition is having a growth-limiting effect or there are other mechanisms of action responsible for 5-FU effect in HT-29 cells remains unresolved.

Exogenous thymidine can have a number of effects on 5-FU cytotoxicity, by acting at several sites along its anabolic and catabolic pathways. Firstly, thymidine can reverse 5-FU cytotoxicity by releasing cells from dTTP depletion due to TS inhibition (Umeda and Heidelberger, 1968). 20μM thymidine reversed the growth inhibitory effects of 5-FU on H630 human adenocarcinoma cells (Chu, 1990). Cell growth was restored to 80% of control levels when thymidine was combined with 5-FU and IFN, a combination which produced 38% inhibition in the absence of thymidine. Elias and Crissman also demonstrated that thymidine blocked the enhanced TS inhibition caused by synergy between IFN and 5-FU resulting in an 8-fold increase in 5-FU IC_{50} (Elias & Crissman, 1988).

Secondly, thymidine facilitates increased incorporation of 5-FU into RNA resulting in enhanced cytotoxicity (Speigelman, 1980; Kufe and Egan, 1981; Martin, 1978; 1980, Akazawa, 1986). The mechanism is thought to be competition for TP, thus controlling the relative incorporation into RNA and DNA (Figure 1.1). Thirdly, thymidine can also be converted to dTTP which acts as a feedback inhibitor of ribonucleotide reductase suppressing the production of FdUTP and also competing with FdUTP for DNA polymerase (Akayawa, 1986). Fourthly, thymine produced from catabolism of thymidine, may compete with 5-FU for DPD the first enzyme in the

catabolism pathway of 5-FU, resulting in an extension of the half-life of 5-FU. Finally as mentioned earlier thymidine can act as a donor of the deoxyribose moiety required for TP activity and subsequent 5-FdUMP formation.

Studies were carried out to assess the effects of thymidine on 5-FU cytotoxicity in HT-29, HT-29(V) and HT-29(TP) cells. Cells were treated concurrently with 5-FU and thymidine (10 and 100μM). In a separate experiment, cells were treated with 5-FU alone and thymidine was introduced into the medium in the recovery period. The aim of these experiments was to establish whether TS inhibition was contributing to 5-FU cytotoxicity (illustrated by a reversal of 5-FU toxicity) or whether other mechanisms of action such as RNA toxicity predominate (illustrated by an augmentation of 5-FU effect).

6.2 Materials and Methods

6.2.1 Chemicals and reagents

See section 2.4.2.1. Deoxyinosine was purchased from Aldrich (Poole, UK). Dialysed serum, Leucovorin (Folinic acid) and Thymidine were purchased from SIGMA (Poole, UK).

6.2.2 Deoxyinosine

6.2.2.1 Method

The MTT assay was used to assess 5-FU cytotoxicity as described in section 2.4.2.2. Cells were treated with 5-FU ($2.5x10^{-7}$-$5x10^{-5}$M) simultaneously with deoxyinosine (150 or 300 μM). In all experiments one half of each 96 well plate was treated with 5-FU alone under normal conditions as a control. Following drug treatment cells were allowed to recover for 72 hours (section 2.4.2.2) and assessed for growth inhibition.

6.2.3 Dialysed serum

6.2.3.1 Method

The MTT assay was used to assess 5-FU cytotoxicity as described in section 2.4.2.2. Four experiments were carried out to assess the effects of pyrimidines in the growth medium during the growth inhibition assay. Cells were seeded in 96-well -plates (500 cells/well) treated with increasing concentrations of 5-FU ($2.5x10^{-7}$ to $5x10^{-5}$M), and then allowed to recover for 3 days, all in the presence of dialysed serum containing medium. Alternatively, cells were seeded and treated with 5-FU in dialysed serum containing medium but allowed to recover in normal serum containing medium. In addition, the effects of 24 and 72 hour 5-FU exposure were assessed.

6.2.4 Leucovorin

6.2.4.1 Method

The MTT assay was used to assess 5-FU cytotoxicity as described in section 2.4.2.2. Two experiments were carried out to assess the effects of Leucovorin pre-treatment. In both experiments, cells were pre-treated with LCV (10μM) for 24 hours. In the first experiment, following LCV pre-treatment, cells were subsequently treated with 5-FU ($2.5x10^{-7}$-$5x10^{-5}$M). In the second experiment, cells were incubated in drug-free medium for 6 hours prior to 5-FU treatment. In both cases, one half of each 96 well plate was treated with 5-FU alone under normal conditions as a control. Following drug treatment, cells were allowed to recover for 72 hours in drug-free medium (section 2.4.2.2) and assessed for growth inhibition.

6.2.5 Thymidine

6.2.5.1 Method

The MTT assay was used to assess 5-FU cytotoxicity as described in section 2.4.2.2. Four experiments were carried out to assess the effects of thymidine on 5-FU

cytotoxicity. Cells were treated with 5-FU and thymidine (10 or 100μM) simultaneously for 24 hours. Alternatively, cells were treated with 5-FU for 24 hours followed by thymidine (10 or 100μM) for 72 hours. In all experiments one half of each 96 well plate was treated with 5-FU alone under normal conditions as a control.

6.3 Results

6.3.1 Deoxyinosine

5-FU cytotoxicity was evaluated in HT-29, HT-29(V) and HT-29(TP) cells in the presence of the deoxyribose donor, deoxyinosine. There was no increase in 5-FU cytotoxicity in HT-29, HT-29(V) and HT-29(TP) cells treated in the presence of either 150 or 300μM deoxyinosine (Figure 6.1 and Table 6.1). Deoxyinosine increased the resistance of the cells to 5-FU as seen by an increase in 5-FU IC_{50} compared with 5-FU alone. In particular, there was a statistically significant increase in 5-FU IC_{50} when HT-29(TP) cells were treated with 150 and 300μM deoxyinosine compared with 5-FU alone ($p<0.01$, Students' t-test).

Cell Line	5-FU Alone	Deoxyinosine (150μM)	Deoxyinosine (300μM)
HT-29	20.2±7.5 n=3	33.1±13.6 n=3	17.9±0.6 n=3
HT-29(V)	9.6±2.5 n=3	14.3±10.9 n=3	24.8±10 n=3
HT-29(TP)	8.7±1.0 n=3	13.9±2.0 n=3	19.1±7.6 n=3

Table 6.1 Effect of Deoxyinosine on 5-FU cytotoxicity in HT-29, HT-29(V) and HT-29(TP) cells HT-29 cells. Growth inhibition was evaluated using the MTT Assay. Cells were seeded in 96-well plates and treated with increasing concentrations of 5-FU and a fixed concentration of Deoxyinosine (150 or 300μM) for 24 hours. Within each 96-well plate, one half was treated with 5-FU alone, no LCV pre-treatment, as a control.

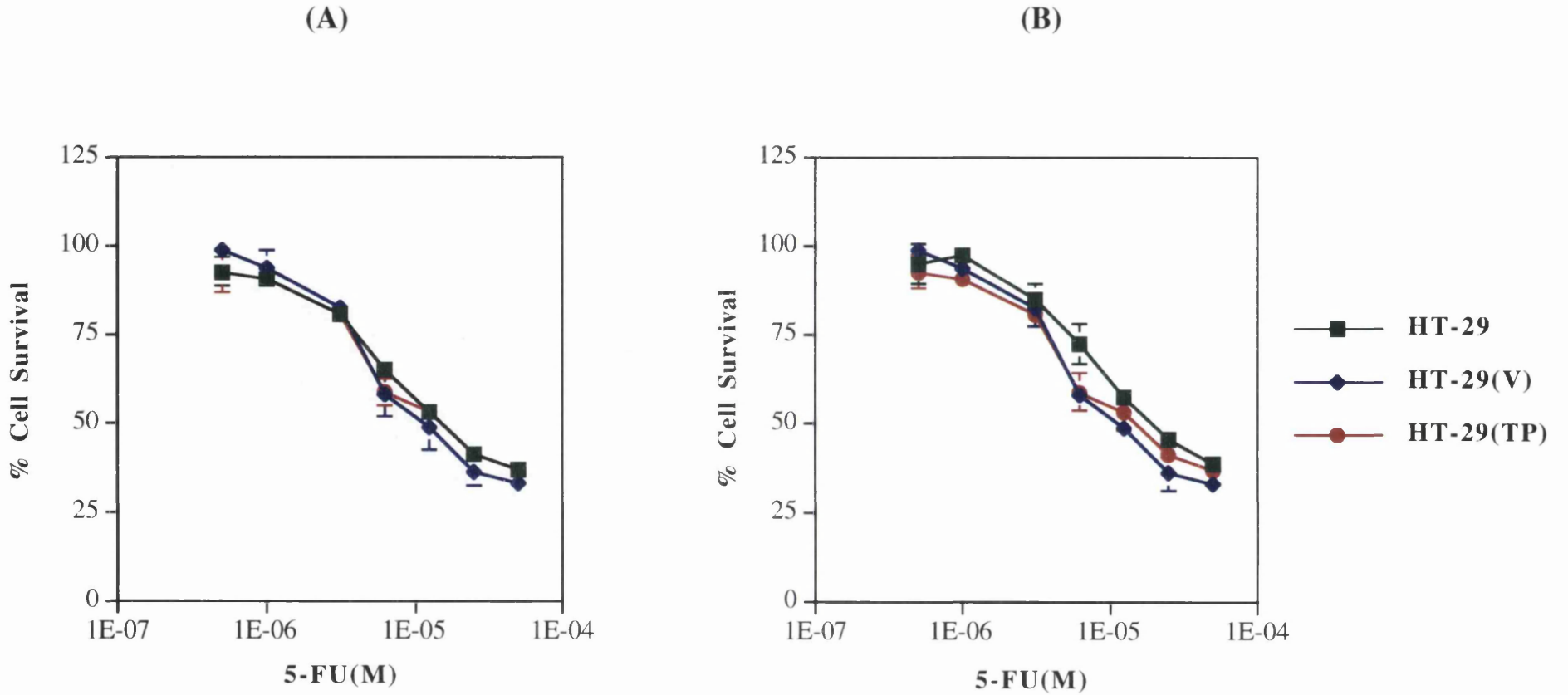


Figure 6.1 Effect of Deoxyinosine, a dR-1-P donor, on 5-FU cytotoxicity. HT-29, HT-29(V) and HT-29(TP) cells were treated with 5-FU for 24 hours in the presence of Deoxyinosine; 150μM, **(A)** and 300μM, **(B)**. Growth inhibition was measured in 96well plates, (500 cells/well in Ham's F10/DMEM medium containing dialysed foetal bovine serum). Increasing oncentrations of 5-FU were added after allowing cell to attach adn grow for 48 hours. Cell growth was measured by incubation with MTT (50μg/mL) for 4 hours. Within each 96-well plate, one half was treated with 5-FU alone as a control. Error bars represent the standard error of means from three 96-well plates, with 4 wells per plate.

6.3.2 Dialysed serum

5-FU cytotoxicity under conditions of dialysed serum-containing medium was evaluated by the MTT assay. Twenty-four and 72-hour drug exposure times were assessed. Dose-response curves are illustrated in Figures 6.2 and 6.3 for dialysed serum and dialysed serum followed by normal serum respectively. Associated 5-FU IC_{50}'s are outlined in Tables 5.2 and 5.3.

There was no increase in 5-FU cytotoxicity in HT-29, HT-29(V) or HT-29(TP) cells in the presence of dialysed serum containing medium compared with normal serum containing medium. The presence of dialysed serum containing medium appears to increase the resistance of the cells to 5-FU (24 and 72 hour exposures). For example, there was a statistically significant increase in 5-FU IC_{50} in HT-29, HT-29(V) and HT-29(TP) cells when dialysed serum was present following 72 hour drug treatment ($p<0.001$, Students' t-test, Table 6.2). When dialysed serum was present in medium during drug treatment only, a similar trend of increased resistance to 5-FU was observed (Table 6.3).

Cell Line	5-FU IC_{50} (μM)			
	24 hours		72 hours	
	5-FU Alone	Dialysed serum	5-FU Alone	Dialysed serum
HT-29	18.9±1.4 n=3	29.4±2.2 n=3	3.9±0.5 n=3	7.0±0.3 n=3
HT-29(V)	21.4±2.4 n=3	35.7±4.0 n=3	3.9±1.6 n=3	6.5±0.4 n=3
HT-29(TP)	21.3±2.3 n=3	26.9±2.1 n=3	4.5±0.4 n=3	6.5±0.3 n=3

Table 6.2 Effect of dialysed serum, present throughout the MTT assay, on 5-FU cytotoxicity in HT-29, HT-29(V) and HT-29(TP) cells. Growth inhibition was measured using the MTT Assay. Dialysed serum present throughout assay. IC_{50}'s are means and standard errors of 3 plates, 8 wells per plate for each concentration of 5-FU.

Cell Line	5-FU IC_{50} (μM)			
	24 hours		72 hours	
	5-FU Alone	Dialysed/Normal serum	5-FU Alone	Dialysed/Normal serum
HT-29	18.9±1.4 n=3	21.8±4.3 n=3	3.9±0.5 n=3	4.6±0.4 n=3
HT-29(V)	21.4±2.4 n=3	37.6±5.2 n=3	3.9±1.6 n=3	6.5±0.6 n=3
HT-29(TP)	21.3±2.3 n=3	24.1±0.2 n=3	4.5±0.4 n=3	5.2±0.3 n=3

Table 6.3 Effect of dialysed serum followed by normal serum containing medium, during an MTT assay, on 5-FU cytotoxicity in HT-29, HT-29(V) and HT-29(TP) cells. Growth inhibition was measured using the MTT Assay. Dialysed serum was present during seeding of cells and 5-FU treatment. During recovery period normal serum-containing medium was used. IC_{50}'s are means and standard errors of 3 plates, 8 wells per plate for each concentration of 5-FU.

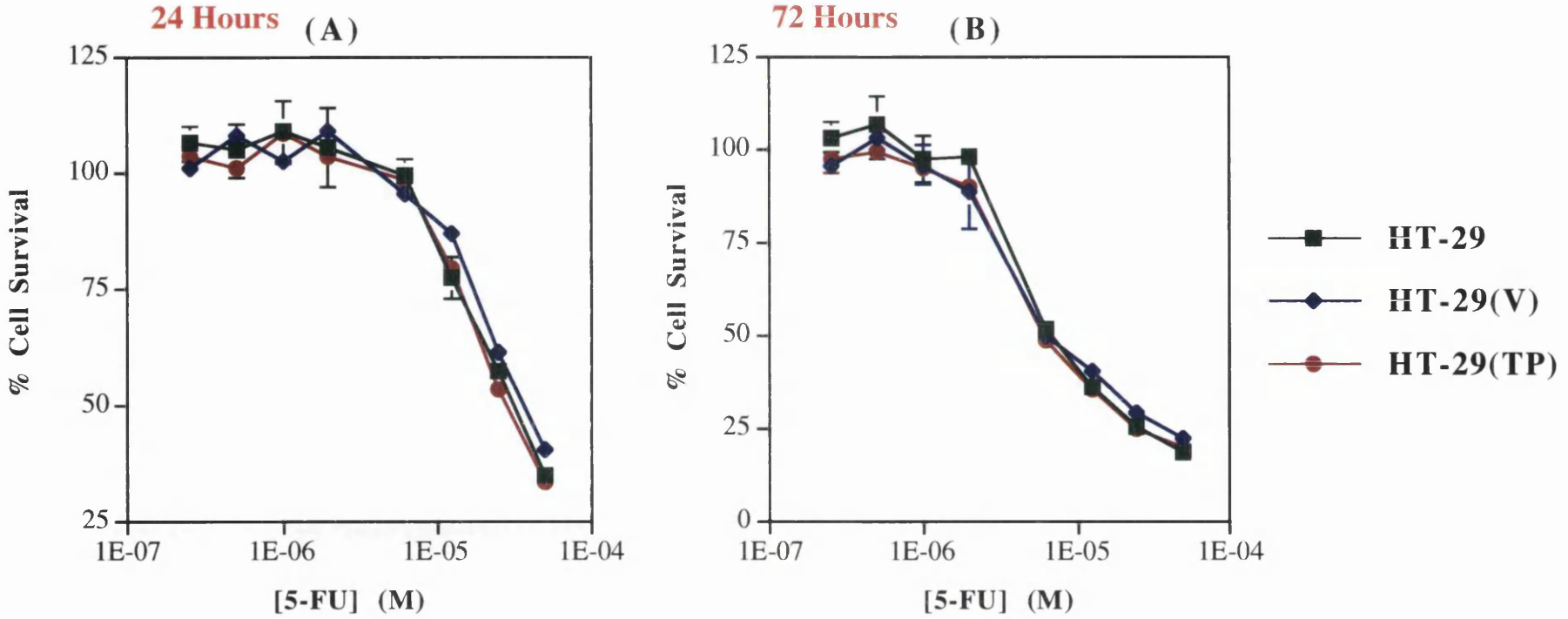


Figure 6.2 Effect of dialysed serum containing present throughout the MTT assay. HT-29, HT-29(V) and HT-29(TP) cells were treated with 5-FU for 24, **(A)** and 72 hours, **(B)**. Growth inhibition was measured in 96-well plates, (500 cells/well in Ham's F10/DMEM medium containing dialysed foetal bovine serum). Increasing concentrations of 5-FU were added after allowing cells to attach and grow for 48 hours. Cell growth was measured by incubation with MTT (50μg/mL) for 4 hours. Dialysed serum was present in the medium throughout the assay procedure. Within each 96-well plate, one half ws reated in the presence of normal serum-containing medium throughout the assay as a control. Error bars represent standard error of means of three 96-well plates, with 8 wells per plate.

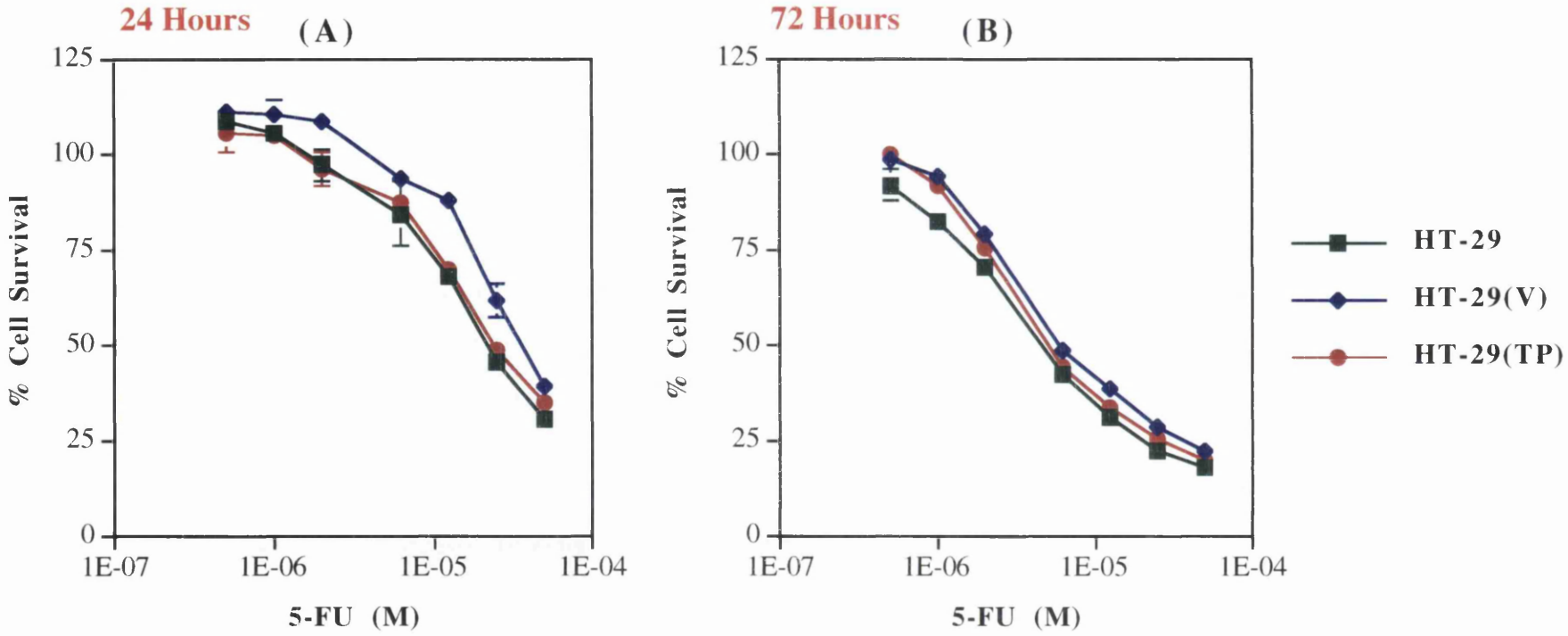


Figure 6.3 Effect of dialysed serum containing medium followed by normal serum containing medium on 5-FU cytotoxicity. HT-29, HT-29(V) and HT-29(TP) cells were treated with 5-FU for 24, (**A**) and 72 hours, (**B**). Growth inhibition was measured in 96-well plates, (500 cells/well in Ham's F10/DMEM medium containing dialysed foetal bovine serum). Increasing concentrations of 5-FU were added in medium containing dialysed serum after allowing cells to attach and grow for 48 hours. Cell growth was measured by incubation with MTT for 4 hours following a period of recovery in normal serum containing medium(drug-free). Error bars represent standard error of means of three 96-well plates, with 8 wells per plate.

6.3.3 Leucovorin

Pre-treatment of HT-29, HT-29(V) and HT-29(TP) cells with Leucovorin (LCV) to expand intracellular pools of reduced folates and increase 5-FU cytotoxicity was evaluated. Cells were treated with LCV (10μM) for 24 hours followed by immediate 5-FU treatment for 24 hours or a 6-hour delay before 5-FU treatment.

Pre-treatment with LCV followed by 5-FU treatment did not result in a statistically significant difference in 5-FU IC_{50} ($p>0.1$, Students' t-test) in HT-29 cells and HT-29(TP) cells compared to 5-FU alone. Similarly, when a 6 hour delay was included for folate polyglutamation, there was no significant difference in 5-FU IC_{50} ($p>0.1$, Students' t-test) compared with 5-FU alone. There was a statistically significant decrease (1.4-fold) in 5-FU cytotoxicity in HT-29(V) cells when LCV was administered prior to 5-FU without a delay compared with 5-FU alone ($p<0.01$, Students t-test). This was not significant when a 6 hour delay was included ($p>0.1$, Students t-test).

Cell Line	**5-FU IC_{50} (μM)**		
	5-FU Alone	**5-FU + Leucovorin (No delay)**	**5-FU + Leucovorin (6 hour delay)**
HT-29	12.2±1.2	12.1±0.9	14.8±2.8
HT-29(V)	19.1±1.8	13.9±0.9	18.6±1.4
HT-29(TP)	15.8±1.9	14.4±2.7	18.3±7.5

Table 6.4 Effect of Leucovorin pre-treatment on 5-FU cytotoxicity in HT-29, HT-29(V) and HT-29(TP). Growth inhibition was evaluated using the MTT Assay. Cells were seeded in 96-well plates and treated with Leucovorin (LCV) for 24 hours. Following LCV pre-treatment cells were either treated with increasing concentrations of 5-FU immediately or incubated in drug free medium for 6 hours prior to treatment 5-FU. Within each 96-well plate, one half was treated with 5-FU alone, no LCV pre-treatment, as a control. IC_{50}'s are means and standard errors of three 96-well plates, four wells per plate for each 5-FU concentration.

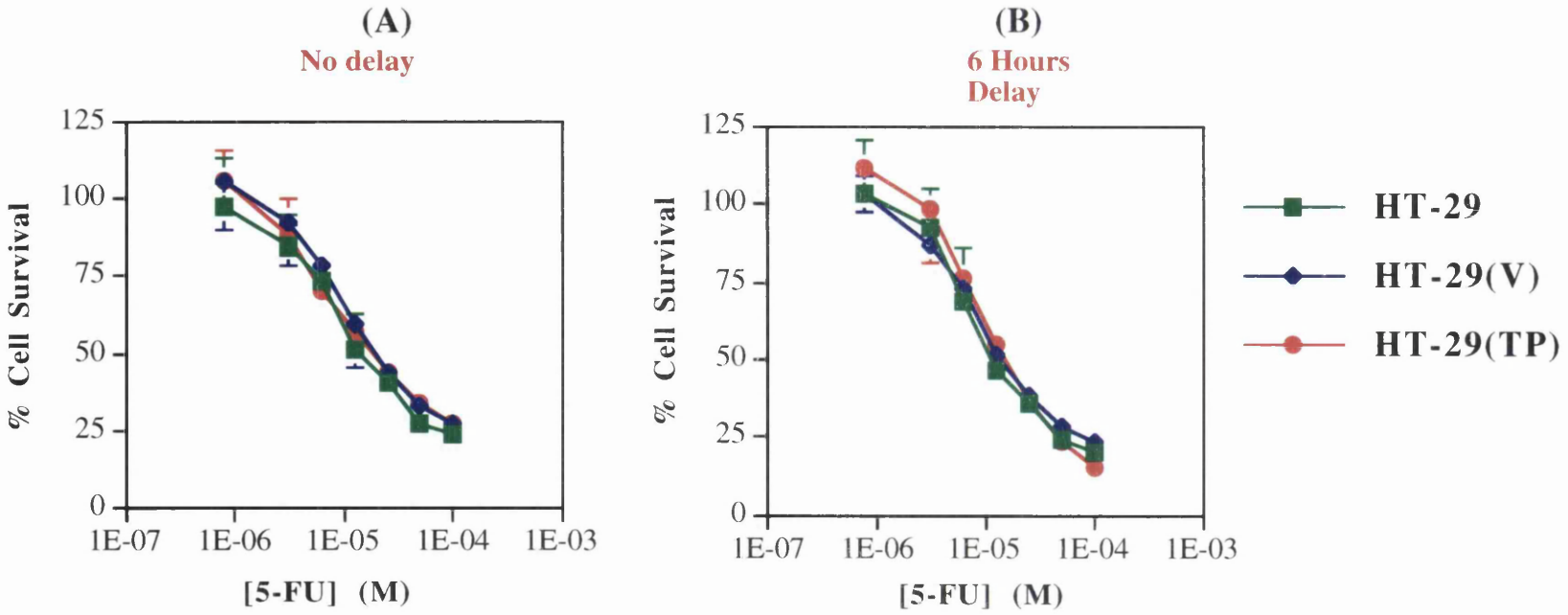


Figure 6.4 Effect of Leucovorin on 5-FU cytotoxicity in HT-29 cells transfected with TP, compared with HT-29 transfected with the vector alone, HT-29(V) and the parental cells, HT-29. Cytototoxicity was measured in 96-well plates using the MTT Assay. Cells were pre-treated with LCV(10μM) for 24 hours, after which time, cells were either treated immediately with increasing concentrations of 5-FU for 24 hours, **(A)** or after a 6 hour delay in drug-free medium **(B)**. Growth inhibition was measured after a further 72 hours in drug-free medium when cells were incubated with MTT (50μg/mL) for 4 hours. Within each 96-well plate cells, one half was treated with 5-FU alone, no LCV pre-treatment as a control. IC_{50}'s are outlined in Table 11.

6.3.4 Thymidine

5-FU cytotoxicity was evaluated in HT-29, HT-29(V) and HT-29(TP) cells in the presence of exogenous thymidine (10 or 100μM). There was no increase in 5-FU IC_{50} in HT-29 and HT-29(TP) cells when treated with thymidine (10 or 100μM) compared with 5-FU alone. In particular, there was no statistically significant difference in 5-FU IC_{50} when HT-29(TP) cells were treated with 5-FU and thymidine (10 or 100μM) compared with 5-FU alone ($p>0.1$, Students' t-test). There was a significant increase (1.4-fold) in 5-FU IC_{50} in HT-29(V) cells treated with 10μM thymidine ($p<0.05$, Students' t-test) but this was not significant following 100μM thymidine ($p>0.1$, Students' t-test). Dose response curves are illustrated in Figure 6.5 and associated IC_{50} values are shown in Table 6.5.

Cell Line	**5-FU IC_{50} (μM)**		
	5-FU Alone (no thymidine)	**Thymidine (10μM)**	**Thymidine (100μM)**
HT-29	7.2±0.1 n=3	5.7±1.0 n=3	5.6±1.1 n=3
HT-29(V)	7.8±0.2 n=3	11.3±2.7 n=3	10.6±4.9 n=3
HT-29(TP)	8.8±0.6 n=3	8.0±1.7 n=3	8.8±0.9 n=3

Table 6.5 Effect of thymidine on 5-FU cytotoxicity in HT-29, HT-29(V) and HT-29(TP) cells. HT-29 parental cells, HT-29 transfected with TP, HT-29(TP)and HT-29 transfected with the vector alone were treated with increasing concentrations of 5-FU and either 10 or 100μM thymidine simultaneously for 24 hours. One half of each plate was treated with 5-FU alone as a control. Growth inhibition was measured using the MTT assay and IC_{50}'s are means and standard errors of 3 96-well plates with 4 wells per plate.

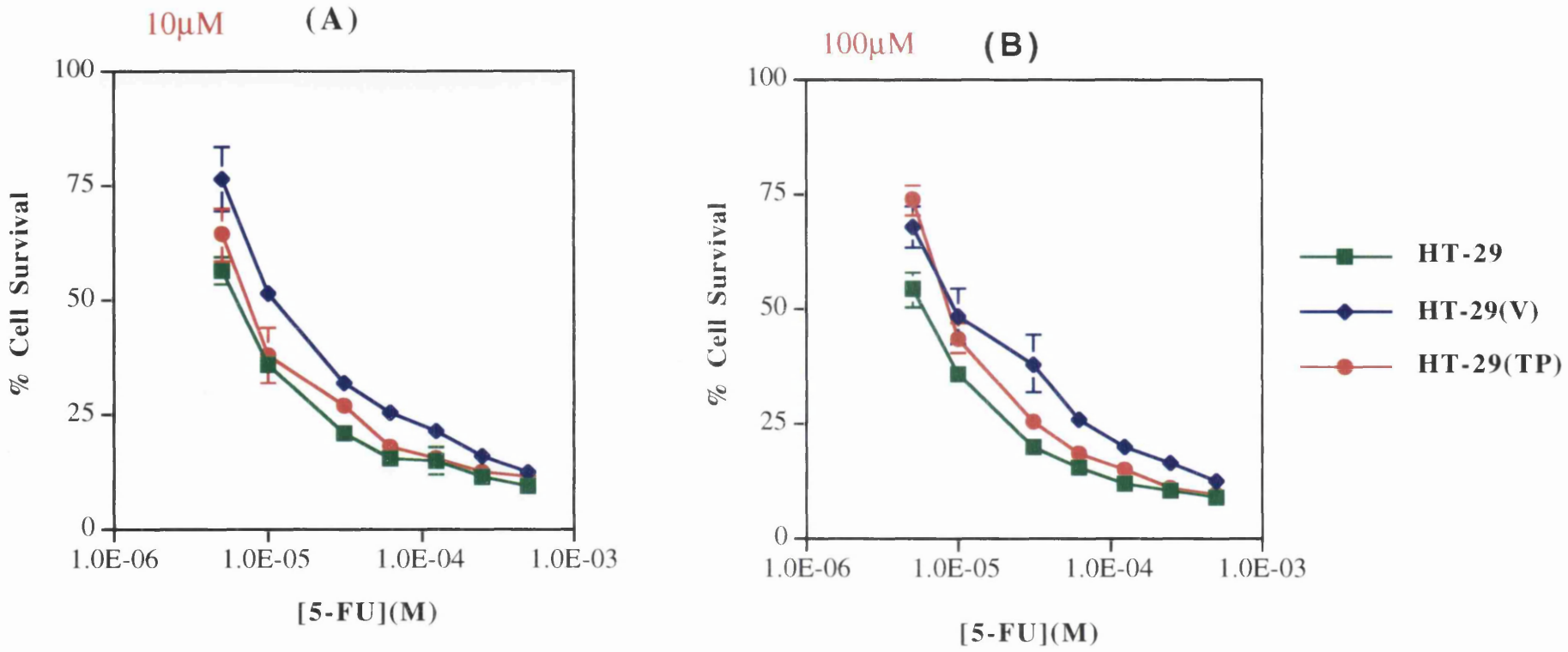


Figure 6.5 Effect of Thymidine on 5-FU cytotoxicity in HT-29(t) cells. HT-29 and HT-29(TP) cells were treated with 5-FU for 24 hours in the presence of Thymidine; 10μM, **(A)** and 100μM, **(B)**. Growth inhibition wasmeasured in 96well plates, (500 cells/well in Ham's F10/DMEM medium containing foetal bovine serum). Increasing concentrations of 5-FU were added after allowing cell to attach and grow for 48 hours. Cell growth was measured by incubation with MTT (50μg/mL) for 4 hours. Within each 96-well plate, one half was treated with 5-FU alone as a control. Error bars represent the standard error of means from three 96-well plates, with 4 wells per plate.

6.3.5 5-FU and Thymidine Post-treatment

HT-29, HT-29(V) and HT-29(TP) cells were treated with increasing concentrations of 5-FU for 24 hours followed by thymidine (10 or 100μM) in the recovery period. There was no statistically significant difference in 5-FU cytotoxicity when HT-29(TP) cells were treated with thymidine following 5-FU treatment ($p>0.1$, Students' t-test). There is however, a statistically significant increase in 5-FU IC_{50} in HT-29 (1.3-fold) and HT-29(V) cells (2-fold) following 100μM thymidine post-treatment compared with 5-FU alone ($p<0.01$ and $p<0.002$ respectively, Students' t-test).

Cell Line	**5-FU IC_{50} (μM)**		
	5-FU Alone	**Thymidine (10μM)**	**Thymidine (100μM)**
HT-29	5.2±0.5	5.3±0.4	6.7±0.3
HT-29(V)	7.5±1.2	11.9±5.9	15.3±2.2
HT-29(TP)	5.4±0.5	6.4±0.9	6.1±0.7

Table 6.6 Effect of thymidine post-treatment on 5-FU cytotoxicity in HT-29, HT-29(V) and HT-29(TP) cells. HT-29 parental cells, HT-29 transfected with TP, HT-29(TP)and HT-29 transfected with the vector alone were treated with increasing concentrations of 5-FU for 24 hours followed by either 10 or 100μM thymidine for 72 hours. On one half of each plate was treated with 5-FU alone as a control. Growth inhibition was measured using the MTT assay and IC_{50}'s are means and standard errors of 3 96-well plates with 4 wells per plate.

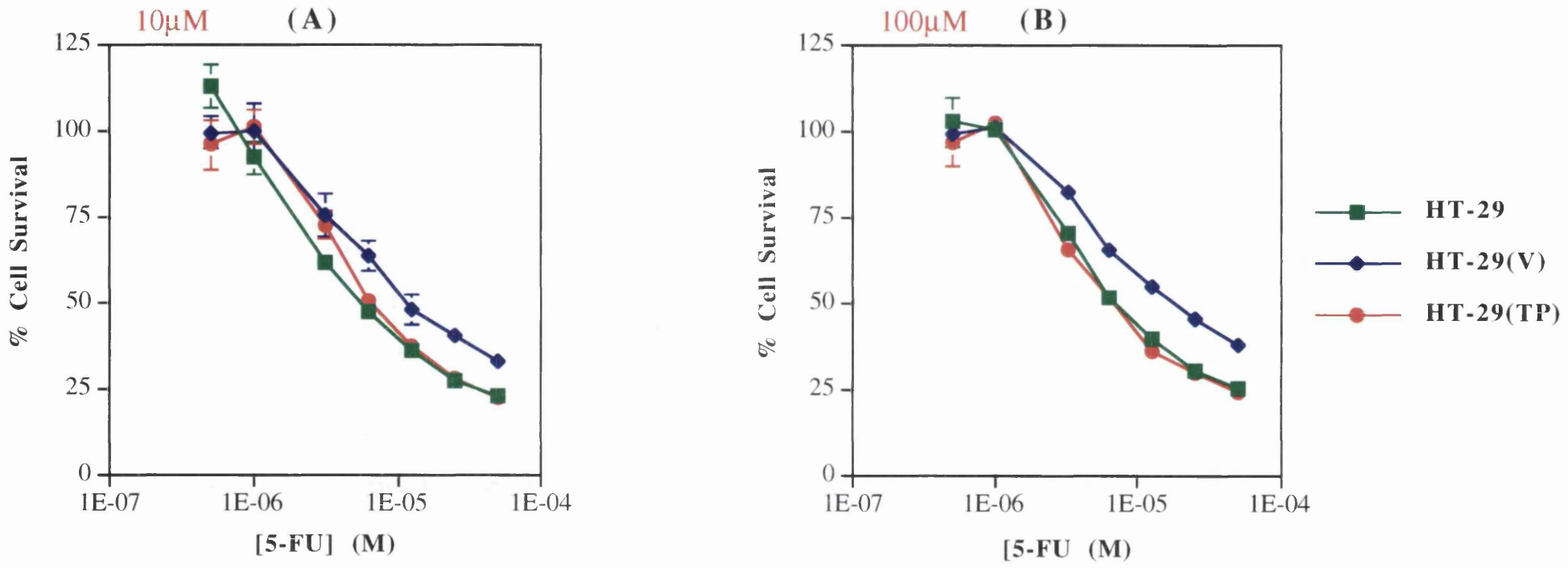


Figure 6.6 Effect of Thymidine post-treatment on 5-FU cytotoxicity in HT-29(t) cells. HT-29, HT-29(V) and HT-29(TP) cells were treated with 5-FU for 24 hours followed by Thymidine; 10µM, **(A)** or 100µM, **(B)** for 72 hours. Growth inhibition wasmeasured in 96well plates, (500 cells/well in Ham's F10/DMEM medium containing foetal bovine serum). Increasing concentrations of 5-FU were added after allowing cell to attach and grow for 48 hoursand thymidine was included in the medium during the 72 hour recovery phase. Cell growth was measured by incubation with MTT (50µg/mL) for 4 hours. Within each 96-well plate, one half was treated with 5-FU alone as a control. Error bars represent the standard error of means from three 96-well plates, with 4 wells per plate.

6.4 Discussion

The anabolism of 5-FU to 5-FdUrd and 5-FdUMP and subsequent inhibition of TS is influenced by a number of variables. In the present study, an increase in expression of the first anabolic enzyme in this pathway, TP, did not increase TS inhibition although overall cytotoxicity was increased 1.6-fold in cells transfected with TP compared with vector alone controls. Therefore the response of the cells to the addition or removal of potentially rate-limiting factors was explored.

Firstly, levels of pyrimidines in the culture medium during cytotoxicity experiments do not appear to compete with 5-FU for TP, since on removal no increase in cytotoxicity between HT-29, HT-29(V) and HT-29(TP) was observed. Indeed, removal of pyrimidines from the cell culture medium during cytotoxicity assays results in increased resistance to 5-FU. This may be due to a slowing down of the growth rate of the cells as a result of a reduction in nutrients in the culture medium required for optimal growth. In retrospect, it would have been better to maintain the cells in dialysed serum for 3-4 weeks prior to the cytotoxicity experiments to allow them to become accustomed to the change in growth conditions.

Likewise 2'-deoxyinosine (150 and 300μM) did not enhance 5-FU cytotoxicity in HT-29, HT-29(V)or HT-29(TP) cells, indicative that dR-1-P levels are not rate-limiting. Interestingly, 2'-deoxyinosine did also increase the resistance of the HT-29(TP) cells to 5-FU. An explanation for this may be that increased TP and dR-1-P cause an increase in the levels of dUrd which, upon further anabolism to dUMP result in competition with 5-FdUMP for TS and instability of the ternary complex.

The levels of reduced folates required for stabilisation and retention of the ternary complex between 5-FdUMP and TS were also explored. HT-29, HT-29(V) and HT-29(TP) cells were pre-treated with LCV (10μM) in order to expand the intracellular

concentrations of the reduced folates. 24-hour pre-treatment of cells with LCV did not result in greater 5-FU cytotoxicity in HT-29 or HT-29(TP) cells. In addition, when further polyglutamation of reduced folates was permitted by a 6-hour interval between LCV and 5-FU treatments, 5-FU cytotoxicity remained unaltered. There was a 1.4 fold decrease in 5-FU cytotoxicity with LCV treatment compared with 5-FU alone in HT-29(V) cells suggesting a minor role for TS inhibition in these cells. This difference is not significant when a 6 hour interval is introduced between treatments however. Therefore, it would appear that sub-optimal concentrations of reduced folates are not limiting TS inhibition and subsequent toxicity in these cell lines. Another possible explanation is, however, that the cells were unable to transport LCV intracellularly. Low affinity, high capacity folate transport has been demonstrated in Methotrexate-resistant cells (Boarman, 1992). Alternatively, the ability of folylpolyglutamate synthetase to convert reduced folates to the more potent polyglutamated state may be impaired (Romanini, 1991). These factors were not assessed in the current study therefore no firm conclusions about the role of these systems in the cytotoxicity of 5-FU in HT-29 cells could be made.

If 5-FU toxicity in HT-29 cells results from TS inhibition and dTTP depletion, then exogenous thymidine should reverse these effects. Examples of complete protection from 5-FU cytotoxicity by thymidine have been reported (Madoc-Jones, 1968 and Murgo, 1980). In the present study thymidine (10 and 100μM) was administered concurrently or following 5-FU treatment. No change in cytotoxicity was observed in HT-29(TP) cells treated with thymidine and 5-FU compared with 5-FU alone. Thymidine (100μM) post-treatment did however increase 5-FU IC_{50} values in HT-29 and HT-29(V) cells (1.3-fold and 2-fold respectively) suggesting a minor role for TS inhibition in these cells. Previous studies demonstrated inhibition of TS in HT-29 cells by Western immunoblotting (Figures 4.7 and 4.8). The lack of effect in HT-29(TP) cells

would indicate that increased TP activity in HT-29(TP) cells may be reversing the capacity of thymidine to rescue cells from 5-FU by the phosphorolytic cleavage of thymidine to thymine rendering it unavailable to bypass TS inhibition. These results indicate that TS inhibition is not, however, the principle mechanism of action of 5-FU in these cells and that formation of 5-FdUMP may not be critical.

These data together suggest that, although increased TP expression enhances the cytotoxicity of 5-FU 1.6-fold in HT-29(TP) cells compared with HT-29(V), the cytotoxicity of 5-FU cannot be further enhanced by the addition of LCV or deoxyinosine. In addition, TS inhibition does not appear to be the principle mechanism of 5-FU cytotoxicity in HT-29 parental and transfected cells although a minor role was apparent. Further experiments would be required to determine whether RNA or DNA toxicity or both contribute to 5-FU growth inhibition in these cells.

It is worth noting that, as with the dialysed serum experiments, different results may have been obtained in these studies if the cells had been maintained in dialysed serum for 3-4 weeks prior to the cytotoxicity experiments. The true effect of exogenous leucovorin on 5-FU cytotoxicity may have been masked by the presence of folates in normal serum containing medium and exogenous thymidine may have reversed 5-FU cytotoxicity.

Cytotoxicity studies *in vitro* are currently underway in the Department of Medical Oncology at the University of Glasgow. Preliminary data has indicated that HT-29(TP) cells are more sensitive to 5'-dFUrd than HT-29(V) and HT-29 (IC_{50}'s: 50.82± 1.11μM; 93.77±2.21μM; 71.5±13.9μM respectively, data unpublished). Interestingly, HT-29(V) cells are more resistant to 5'dFUrd than the parental cells as seen in the present study. In addition, xenograft studies in mice will be conducted to assess the impact of increased TP on 5-FU and it's prodrugs *in vivo*.

CHAPTER 7

7. General Discussion

7.1.1.1 Modulation of 5-FU

Despite being the most commonly used anti-cancer agent for the treatment of advanced colorectal carcinoma, 5-FU has only modest anticancer effects. In combination with biochemical modulators however, such as LCV, the efficacy of 5-FU is markedly enhanced both *in vitro* and clinically (Machover, 1986 and Poon, 1991). Nevertheless, no overall improved survival rate has been demonstrated.

Early *in vitro* studies in mouse and human cell lines demonstrated that the uptake of 5-FU through the plasma membrane is not rate-limiting in it's cytotoxic action (Laskin, 1979). Rather, the intracellular metabolism of 5-FU is responsible for the varied sensitivities of the cell lines to 5-FU. In particular, the activities of the activating enzymes TP, UP and ORTPase were consistently higher in the more sensitive cell lines. Similarly, a human ovarian cell line resistant to 5-FU (PEO4) had reduced TP activity compared with cells derived from the same tumour prior to the onset of resistance (Chu, 1990). In addition, drug-induced resistance to 5-FU is associated with lower activities of both activating enzymes TP and UP, in L1210 leukemia and Ehrlich ascites carcinoma cells (Reichard, 1959).

Recombinant human interferons synergistically enhance the cytotoxic effects of 5-FU *in vitro*, however clinical trials in patients with colorectal cancer have been contradictory and in some cases IFN-α treatment has resulted in greater toxicity and sometimes death compared with 5-FU alone (Corfu-A Study Group, 1995). Studies *in vitro* demonstrated that IFN-α caused an increase in the activity of the 5-FU anabolic enzyme TP and increased levels of the cytotoxic nucleotide 5-FdUMP which inhibits TS (Schwartz, 1992).

Aims

The aims of this thesis, which were set out at the beginning of the project, have largely been achieved. The aims were as follows:

1. To characterise a panel of colon adenocarcinoma cell lines with respect to growth kinetics, TP and TS protein expression and intrinsic sensitivity to 5-FU.
2. To evaluate TP protein expression in human colon tumour and normal biopsy tissues.
3. To transfect human colon carcinomas cells *in vitro* with the gene for TP and assess its contribution alone to 5-FU toxicity.
4. To develop a method for the measurement of TP enzyme activity in cell lines and tissues.

The results of this thesis have contributed towards a greater understanding of the role of TP in colon adenocarcinoma cell lines and biopsy tissues and also towards the future application of a gene-directed approach to it's treatment.

Colon Adenocarcinoma Cell Lines

Five colon adenocarcinoma cell lines (HT-29, BE, CACO-2, DLD-1 and LOVO) were evaluated for TP protein expression by Western immunoblotting and enzyme activity was measured using the assay described in Chapter 5. TP protein was detected in the LOVO cell line only. Similar results were obtained when TP activity was measured in these cell lines. TP activity was measurable in all the colon cell lines. HT-29, BE, CACO-2 and DLD-1 cells had similar low activity (2-2.4 pmoles/min/mg) and LOVO cells had the highest activity (25.0 pmoles/min/mg).

The sensitivity of the colon cell lines to 5-FU was measured using the MTT and clonogenic assays and a 3.3 and 4.1-fold range in 5-FU IC_{50} was demonstrated respectively. The two assays were found to correlate (r=0.88) and the MTT assay alone was used in all subsequent cytotoxicity experiments.

TP activity and protein expression did not appear to correlate with 5-FU cytotoxicity. LOVO cells with the highest TP activity and expression were not the most sensitive cell line to 5-FU whereas HT-29 and CACO-2 cells with low TP activity and no measured TP protein were the most sensitive. This was confirmed when TP activity and 5-FU sensitivity was evaluated in a number of cell lines from other tumour sources. TP activity alone did not determine the sensitivity of cell lines to 5-FU (r=0.28).

TS protein was detected in all 5 colon cell lines with a 5.9-fold range in expression. The sensitivity of the cell lines to 5-FU correlated with TS expression (r=0.87) confirming a role for this enzyme as a target for 5-FU in cell lines. These findings are consistent with other reports where TS levels were higher in 5-FU insensitive than sensitive colon and breast cell lines (Johnston, 1992) and in 5 human gastrointestinal tumour cell lines where TS activity directly correlated with 5-FdUrd cytotoxicity (Washtein, 1981). In addition, the role of TS *in vitro* translates to the clinic where colorectal and gastric tumours with a low mean TS protein level demonstrate an improved response to treatment with 5-FU/LV-based treatment than those with a high mean TS level (Johnston, 1995).

Colon Biopsy Tissues

TP expression and activity were also assessed in colon tumours and adjacent non-neoplastic regions to assess the relative levels of TP and to determine the relevance of the colon cell lines as a model for human tumours. TP protein expression was elevated in colon tumour tissues compared with their normal colon counterparts as was TP enzyme activity. This agrees with current literature where TP expression is elevated in tumours of the stomach, colon, ovary and breast compared to normal regions of the same tissue (Zimmerman, 1964; Yoshimura, 1990; Luccioni, 1994; Pauly, 1977 and Fox, 1997).

Interestingly, TP levels were higher in tissue samples than cell lines, suggesting down regulation of activity *in vitro* possibly as a result of a decreased requirement for the angiogenic activity of TP in cells in culture. Another explanation may be that the tumour biopsy samples comprised stroma and inflammatory infiltrate as well as tumour cells and that inflammatory cells such as macrophages have high TP activity (Fox, 1996).

TP protein expression/activity was heterogeneous in tumours as demonstrated by a 6-fold range in expression and a 4-fold range in activity. Heterogeneity of expression/activity of TP in tumours may explain why only a proportion of patients respond to treatment with 5-FU. As a result, increased expression of TP in tumours through the delivery of the TP gene sequence may overcome this lack of sensitivity. This theory was investigated first *in vitro* using DNA transfection studies in colon cell lines.

TP expression correlates with metastasis in colon and gastric cancers (Takebayashi and Maeda, 1996), with angiogenesis in breast and NSCLC (Toi, 1995, Moghadam, 1995 and Koukourakis, 1997) with poorer survival in gastric cancer (Maeda, 1996), poorer prognosis in colon, gastric and NSCLC (Takebayashi, 1995, Maeda, 1996 and Koukourakis, 1997) and malignancy in bladder and colon cancer. Increased TP expression in tumours therefore confers a more aggressive phenotype. This suggests that a transient expression vector rather than a stable expression vector should be used clinically to elevate TP levels, prior to treatment with 5-FU, to lower the risk of promoting tumour growth.

TP Transfection Studies in HT-29 cells

HT-29 cells with no detectable TP protein and low TP activity, were transfected with TP cDNA and a vector alone control. TP protein was detected in HT-29(TP) cells. TP activity was 5-fold greater in HT-29(TP) cells than both HT-29 and HT-29(V) cells.

There was no change in the growth kinetics of the transfected cells and basal levels of TS expression were also unchanged.

Evaluation of the transfected cells with respect to their sensitivity to 5-FU, demonstrated that HT-29(TP) cells were more sensitive to 5-FU than the HT-29(V) cells (1.6-fold reduction in 5-FU IC_{50}) however there was no difference in sensitivity when compared with the parental cells. Therefore, the vector alone controls were more resistant to 5-FU than the parental cells, despite having similar basal levels of TS enzyme and unchanged growth kinetics. This suggests that transfection alone (without the TP cDNA) may have caused genetic alterations in the cells resulting in increased resistance to 5-FU. Transfection with the TP cDNA included in the DNA vector may have sensitised the cells, returning them to a similar level to that of the parental cells.

In addition to assessing growth inhibition by the MTT assay in the transfected cells, the levels of TS ternary complex formed were assessed following treatment of cells with 5-FU at IC_{10} (10μM) and IC_{50} (50μM) concentrations for 1 and 24 hours. There was no apparent change in the ratio of bound to free TS enzyme in HT-29(TP) compared with HT-29(V) cells and the parental cells. This experiment was carried out only once and therefore no firm conclusions could be drawn concerning differences between cell lines. With both 5 and 10μM treatment however, a similar pattern did emerge after 1 and 24 hours. If TS inhibition is the principle mechanism of cytotoxicity of 5-FU in these cells then a small increase in cytotoxicity (1.6-fold) might not be identifiable using this assay at the time points and drug concentrations used in this experiment. Further studies with a wider time course and a range of 5-FU concentrations may provide further information regarding the extent of TS inhibition.

Following treatment with 5-FU, total TS expression was 2-3 fold higher in HT-29(V) than the parental cells. The apparent greater induction of TS may play a role in the increased resistance to 5-FU compared with the parental cells.

TS inhibition remained unchanged despite an elevation in total TS levels at 24 hours in HT-29(V) and HT-29(TP) cells. This would suggest that adequate intracellular concentrations of 5-FdUMP and reduced folates are present to inhibit TS in the transfected cells. This was confirmed when exogenous LCV (10μM) did not enhance the cytotoxicity of 5-FU in HT-29, HT-29(V) and HT-29(TP) cells. In the same way, an increase in the levels of cofactor for TP by the addition of 2'deoxyinosine did not enhance cytotoxicity suggesting that dR-1-P levels are not limiting the activity of TP and hence the cytotoxicity of 5-FU.

In the present study, thymidine was administered to cells concurrently with 5-FU or post-5-FU treatment. Only a small change in 5-FU cytotoxicity was observed (1.3-2-fold) in HT-29 and HT-29(V) cells suggesting a minor role for TS inhibition in the overall cytotoxicity of 5-FU. There was no change however, in the cytotoxicity of 5-FU in HT-29(TP) cells following thymidine treatment. This suggests that the phosphorolytic activity of TP is reducing the availability of thymidine necessary to reverse TS inhibition or compete with 5-FdUrd for incorporation into DNA.

It may be more appropriate to maintain the cells in dialysed serum-containing medium prior to cytotoxicity experiments with modulators such as Leucovorin and thymidine since the presence of folates and pyrimidines in the medium may mask the effects of these agents on 5-FU cytotoxicity.

TS inhibition leads to depletion of thymidylate (dTMP), a precursor of one of the 4 deoxyribonucleotides (dTTP) necessary for DNA synthesis. As a result of dTTP depletion, FdUTP and dUTP become incorporated into DNA in its place (Yin, 1991, Lonn, 1988). Enzymes such as dUTPase and uracil-DNA glycosylase are responsible for excising these nucleotides from DNA (Ingraham, 1980 and Mauro, 1993) and resistance has been shown to be attributable to increased activities of these enzymes and a significant decrease in the level of 5-FU metabolites in DNA (Chu, 1990). The intrinsic

activity of these enzymes was not evaluated in the present study and may explain the lack of effect of increased TP on 5-FU cytotoxicity seen in HT-29(TP) cells.

The activity of TK, the enzyme responsible for the conversion of 5FdUrd to 5-FdUMP was not measured in the present study. Therefore a possible explanation for the lack of effect of increased TP levels on 5-FU cytotoxicity could be that there is insufficient TK enzyme to deal with the increased formation of 5FdUrd. TK activity could be measured using the activity assay outlined in Chapter 5. This would involve using FdUrd as the substrate and ATP as the cofactor in place of 5-FU and dR-1-P. The formation of FdUMP could be detected by HPLC. This would provide further information relating to the anabolic pathway which leads to inhibition of TS by FdUMP.

The *in vitro* data for HT-29 cells are not necessarily representative of the *in vivo* situation however. In HT-29(TP) cells, TS inhibition is not critical for 5-FU cytotoxicity. TS protein and mRNA expression do however, predict for response to 5-FU/LCV-based chemotherapy in patients with colorectal and gastric cancer (Johnston, 1995). In addition, resistance to 5-FU-based therapy can be a result of acute induction of TS protein as well as stable amplification of TS-specific genes (Washtein, 1984, Berger, 1987, Clark, 1987 and Johnston, 1992).

Following commencement of the present study, Schwartz carried out similar transfections in HT-29 cells using the p^{CMV}-TP-Neo vector (Schwartz, 1995). A number of clones with a range of TP activities were reported (1.8-5.4 fold greater than parental cells) which possessed greater sensitivity to 5-FU than the parental cells and a more significant effect on 5-FU cytotoxicity was demonstrated than in the present study. The clone with 5-fold greater TP activity resulted in a 19-fold reduction in 5-FU IC_{50}.

Comparison of both studies revealed that different drug exposure times and methods for assessing 5-FU cytotoxicity were used. However, it would be expected that

a similar relative difference between transfected and control cells would be obtained despite the differences in methodology.

One possible explanation for the difference in results obtained could be however that the transfected cells in the present study were pooled rather than cloned. Pooled transfectants should more closely reflect the heterogeneity of TP in tumours. The resultant heterogeneity of expression of TP may be masking any increase in 5-FU cytotoxicity in these cells. This was investigated by immunohistochemical analysis of paraffin-embedded cell pellets with the TP antibody (data not shown). LOVO cells were used as a positive control since they have approximately 3-fold greater TP activity than HT-29(TP) cells and 12-fold greater activity than the parental cells. The intensity of staining in LOVO cells however was very low and there was no visible difference between LOVO and HT-29 or HT-29(TP) cells. It was impossible to evaluate heterogeneity of expression using this method.

A number of other studies have investigated the effects of increased TP expression by DNA transfection. Many have been unable to repeat the work of Schwartz and colleagues. MCF-7 breast carcinoma cells with 100-fold greater TP activity following DNA transfection showed no difference in their sensitivity to 5-FU than the parental cells (Patterson, 1995). This level of TP activity may not have enhanced 5-FU cytotoxicity since the mechanism of action of 5-FU in these cells has been shown to be incorporation into RNA (Kufe and Major, 1981). Haraguchi *et al* (1993) transfected KB epidermoid cells with TP and demonstrated unaltered sensitivity to 5-FU. A recent study where lung carcinoma cells were transfected with TP resulted in a clone with 50-fold greater TP activity and 5-FU IC_{50} was decreased 8-fold (Kato, 1997).

Another study looking at the effects of IFN-α on a panel of colon adenocarcinomas (including HT-29), reported that IFN-α increased the activity of TP in 6 out of 8 cell lines, HT-29 cells being one of them (Tevaearai, 1992). An enhancement

of 5-FU cytotoxicity was observed in only one of the 6 cell lines where increased TP activity was observed. There was no increase in the sensitivity of HT-29 cells to 5-FU.

In some of the above studies thymidine mediated reversal of 5-FU cytotoxicity was evaluated. In many cases, where a difference in 5-FU cytotoxicity was observed, thymidine reversed cytotoxicity implicating TS inhibition as the principle mechanism of 5-FU cytotoxicity (in the others this was not assessed). The data therefore varies from one cell line to another, both with IFN-α treatment and DNA transfection as methods of increasing TP activity. This emphasises that different cell lines utilise different pathways of 5-FU effect and that the results reported by Schwartz and colleagues do not represent a trend observed in all colon cancer cell lines. Therefore, in many of the *in vitro* studies TP activity was not critical for the cytotoxicity of 5-FU. This was further demonstrated in the present study where no correlation could be made between TP activity and 5-FU IC_{50} in a range of tumour cell lines. TP activity was heterogeneous within each tumour type. Two groups of cell lines emerged with high and low TP activity. Within each group 5-FU cytotoxicity was heterogeneous.

The IFN-α studies carried out by Schwartz were conclusive however, in that IFN-α caused an induction of TP, increased levels of 5-FdUMP, with no difference in incorporation of 5-FU into DNA and RNA and no difference in UP activity (Schwartz, 1992). Tevaearai and colleagues could not repeat these results in HT-29 cells and this apparent discrepancy may be answered by examining the time course of IFN-α induced changes in TP activity (Tevaearai, 1992) or may suggest that TP is not critical in the HT-29 cells used in this study.

It would appear that cells in which 5-FU utilises this pathway of effect (5-FU to 5-FdUMP via 5FdUrd) will respond to treatment with IFN-α or DNA transfection with the TP gene and those cell lines in which other mechanisms and pathways predominate, an increase in TP activity (even 100-fold) will have no impact. This could be confirmed

by first screening cell lines with thymidine to demonstrate reversal of TS inhibition and the importance of this pathway in the activation of 5-FU to 5-FdUMP. Following this, transfection studies with p^{CMV}-TP-Neo could be conducted to evaluate whether an increase in TP activity would further enhance the TS inhibition already known to be critical.

RNA Toxicity

As was discussed in the Introduction, two other known targets of 5-FU exist, namely RNA and DNA. FUTP competes with UTP for RNA polymerase. Incorporation of FUTP leads to RNA dysfunction. Incorporation of 5-FU in RNA has been demonstrated in HT-29 (Greenhalgh, 1990). Pre-treatment with thymidine (0.5mM) resulted in an increase in 5-FU cytotoxicity (Greenhalgh, 1990). It is thought that thymidine does so by causing an accumulation of thymidine triphosphate (dTTP) which then results in feedback inhibition of ribonucleotide reductase. The high levels of dTTP are also believed to repress the anabolic conversion of 5-FU into deoxy-derivatives thus preserving it for entry into RNA (Speigelman, 1980). It was evident however that thymidine did not enhance 5-FU cytotoxicity by increasing incorporation into RNA in HT-29, HT-29(V) and HT-29(TP) cells. Pre-treatment with thymidine was not evaluated however and may have revealed a role for RNA-directed cytotoxicity.

DNA toxicity

The deoxynucleotides FdUTP/dUTP compete with dTTP for DNA polymerase. Incorporation of FdUTP into DNA results in inhibition of chain elongation and altered DNA stability causing DNA single- and double-strand breaks as a result of attempts to excise fraudulent nucleotides and repair the DNA. Under normal circumstances dUTP levels are virtually undetectable since dUTPase efficiently hydrolyses dUTP to dUMP thereby making uracil misincorporation into DNA an unlikely event (Brynoff, 1978 and Grafstrom, 1978). Following inhibition of TS and subsequent dTTP depletion the ratio

of dUTP/dTTP is increased, more uracil may become incorporated into DNA generating areas of continuous DNA excision/repair and strand breaks (Goulian 1986).

FdUrd treatment of HT-29 cells resulted in DNA damage in the form of single and double strand breaks (Canman, 1993). Sensitivity to 5FdUrd was related to the level of DNA damage. The appearance of single strand breaks preceded that of double-strand breaks consistent with the 5-FU misincorporation/misrepair mechanism which is expected to yield a larger number of single strand breaks prior to the formation of double strand breaks.

The role of these mechanisms of 5-FU cytotoxicity was not evaluated in the present study. It is therefore feasible that 5-FU utilises either of these pathways as it's mechanism of cytotoxicity in HT-29 cells.

Downstream Events

Despite numerous attempts to modulate the activity of 5-FU and other anti-cancer agents, few studies have addressed the genetic factors, which may control resistance to DNA damaging agents. The p53 gene product, known as "the guardian of the genome" (Lane, 1992) plays a vital role in the nature of cellular response to DNA damage. Following exposure of cells to DNA damaging agents such as 5-FU, expression of and stability of p53 is induced and either arrest in the G_1 phase of the cell cycle (allowing repair of DNA damage to occur) or programmed cell death/apoptosis occurs. Normal p53 function can become disrupted by mutation of the gene causing cells to continue proliferating despite DNA damage and leading to further genetic instability. Apoptosis is also regulated by other genes e.g. bcl-2 and bax. bcl-2 is a repressor of programmed cell death and bax homodimerises or heterodimerises with bcl-2 and counters the death repressor activity of bcl-2 (Oltvai, 1993). The ratio of Bcl-2 to bax is thought to determine the susceptibility to death following an apoptotic stimulus. When

bcl-2 is in excess, cells are protected however, when bax is in excess the bax homodimers dominate and cells are susceptible to apoptosis.

Inada and colleagues reported that apoptosis of gastric and colon tumour xenografts was related to 5-FU cytotoxicity (Inada, 1997). Following treatment with 5-FU, TS inhibition was followed by an accumulation of cells in S-phase (due to inhibition of DNA synthesis) and this was followed by apoptotic death (Inada, 1997). HT-29 cells have a mutated p53 gene which may explain in part the resistance of these cells to 5-FU. Mutant p53 results in a continuation of cell proliferation following DNA damage and resistance to 5-FU.

The concept that increased expression of a single enzyme involved in the anabolism of 5-FU would sensitise HT-29 cells to 5-FU may appear in retrospect to be a naïve one. Studies in patients with colorectal tumours have confirmed that TP expression alone does not confer increased sensitivity to 5-FU (Metzger, 1998). The cytotoxicity of 5-FU is dependent on many factors e.g. the levels of anabolic enzymes, the levels of co-factors and co-substrates for these enzymes (e.g. dR-1-P, RIP or 5,10-CH_2THF), mutations occurring in genes encoding target enzymes resulting in reduced affinity of the associated substrate, levels of DNA and RNA repair enzymes, not to mention the multiple routes of anabolism and subsequent sites of action which differ from one tumour to another.

Despite disappointing results with respect to the cytotoxicity of 5-FU, a greater effect may be seen with 5-FU pro-drugs such as 5'-dFUrd and Tegafur which require activation by TP. Preliminary cytoxicity experiments have indicated an increase in sensitivity of HT-29(TP) cells to 5'-dFUrd and this will be further investigated using xenograft studies. The application of a TP as a candidate gene for gene-directed/fluoropyrimidine pro-drug therapy is therefore promising as a means of

improving the current lack of effective treatment for colon cancer and requires further investigation.

8. BIBLIOGRAPHY

Abbruzzese, J. & Levin, B. (1989). Treatment of advanced colorectal cancer. *ClinicalHaematology/Oncology North America.* **3**:135-53.

Akazawa, S., R. Kumai, K. Yoshida, D. Ayusawa, K. Shimizu & Seno, & T. (1986). The cytotoxocity of 5-fluorouracil is due to it's incorporation into RNA not it's inhibiion of thymidylate sytnthase as evidenced by the use of a mouse cell mutant deficient in thymidylate synthase. *Japanese Journal of Cancer Research.* **77**:620-624.

Alley, M., D. Scudiero, A. monks, M. Hursey, M. Czerwinski, D. Fine, B. Abbott, B. Mayo, R. Shoemaker & Boyd, M. (1988). Feasibility of drug screening with panels of human tumour cell lines using a microculture tetrazolium assay. *Cancer Research.* **48**:589-601.

August, D., R. Ottoe & Sugarbaker, P. (1984). Clinical perspectives on human colorectal metastases. *Cancer Metastases Review.* **3**:303-324.

Baker, S., S. Markowitz, E. Fearon, J. Willson & Vogelstein, B. (1990). Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science.* **249**:912-915.

Barbour, K., S. Berger &Berger, S. (1990). Single amino acid substitution defines a naturally occurring genetic variant of human thymidylate synthase. *Molecular Pharmacology.* **37**:515-518.

Berger, S., J. Chung-Her, L. Johnson & Berger, F. (1987). Thymidylate synthase overproduction and gene amplification in fluorodeoxyuridine-resistant human cells. *Molecular Pharmacology.* **28**:461-467.

Berger, S. & Hakala, H. (1984). Relationship of dUMP and FdUMP pools to inhibition of thymidylate synthase by 5-Fluorouracil. *Molecular Pharmacology.* **25**:303-309.

Berger, S., C. Jenh & Johnston, J. (1985). Thymidylate synthase overproduction and gene amplification in fluorodeoxyuridine resistant human cells. *Molecular Pharmacology.* **28**:461-467.

Boarman, D.& Allegra, C(1992). Intracellular metabolism of 5-Formyl Tetrahydrofolate in human breast and colon cell lines. *Cancer Research.* **52**:36-44.

Boland, C. (1993). The Biology of Colorectal Cancer. *Cancer supplement.* **71**:4180-4186.

Bowen, D., R. Diasio & Goldman, I. (1979). Distinguishing between membrane transport and intracellular metabolism of fluorodeoxyuridine in Ehrlich ascites tumor cells by application of kinetic and high performance liquid chromatography techniques. *The Journal of Biological Chemistry.* **254**:5333-5339.

Breitman, T. & Bradford, R. (1964). The induction of thymidine phosphorylase and excretion of deoxyribose during thymine starvation. *Biochemical and Biphsical Research Communications.* **17**:786-791.

Breitman, T. & Bradford, R. (1964). The induction of thymidine phosphorylase and excretion of deoxyribose during thymine starvation. *Biochemical and Biophysical Research Communications.* **17(6)**:786-791.

Bresnick, E. & Thompson, U. (1965). Properties of deoxythymidine kinase partially purified from animal tumours. *Cancer Research.* **240**:3967-3974.

Brynoff, K., R. Eliasson & Reichard, P. (1978). Formation of Okazaki fragments in polyoma DNA synthesis caused by misincorporation of uracil. *Cell.* **13**:573-580.

Cadman, E., L. Davis & Heimer, P. (1979). Enhanced 5-fluorouracil nucleotide formation following methotrexate: biochemical explanation for drug synergism. *Cancer Research.* **39**:4905.

Canman, C., T. Lawrence, D. Shewach, H. Tang & Maybaum, J. (1993). Resistance to fluorodeoxyuridine-induced damage and cytotoxicity correlates with an elevation of deoxyuridine triphosphatase activity and failure to accumulate deoxyuridine triphosphate. *Cancer Research.* **53(21)**:5219-24.

Caradonna, S. & Cheng, Y. (1980). The role of deoxyuridine triphosphate nucleotidohydrolase, uracil-DNA glycolylase and DNA polymerase α in the metabolism of FUdR in human tumour cells. *Molecular Pharamcology.* **18**:513-520.

Carmichael, J., W. DeGraff, A. Gazdar, J. Minna & Mitchell, J. (1987). Evaluation of a Tetrazolium-based Semiautomated Colorimetric Assay: Assessment of Chemosensitivity Testing. *Cancer Research.* **47**:936-942.

Carney, D., A. Gazdar, G. Bepler, J. Giccion, P. Marangos, T. Moody, M. Zweig & Minna, J. (1985). Establishment and identification of small cell lung cancer cell lines having classic and variant features. *Cancer Research.* **45**:2913-2923.

Carrico, C. & Glazer, R. (1979). Augmentation by thymidine of the incorporation and distribution of 5-fluorouracil into ribosomal RNA. *Biochemical and Biophysical Research Communications.* **87**:664-670.

Chen, T. (1978). Evolution *in vitro* of stemlines with minimal karyotypic deviations in a human heteroploid cell line. *Journal of the National Cancer Institute.* **61**:277-284.

Cheng, Y.-C. & Nakayama, K. (1983). Effects of 5-fluoro-2'-deoxyuridine on DNA metabolism in HeLa cells. *Molecular Pharmacology.* **23**:171-174.

Chu, E., J. Drake & Koeller, D. (1991). Induction of thymidylate synthase associated with multidrug resistance in human breast and colon cancer cell lines. *Molecular Pharmacology.* **39**:136-143.

Chu, E., G. Lai, S. Zinn & Allegra, C. (1990). Resistance of a human ovarian cancer cell line to 5-Fluorouracil associated with decreased levels of 5-Fluorouracil in DNA. *Molecular Pharmacology.* **38**:410-417.

Chu, E., D. Voeller & Johnston, P. (1993). Regulation of thymidylate synthase in human colon cancer cells treated with 5-fluorouracil and interferon-γ. *Molecular. Pharmacol.ogy* **43**:527.

Chu, E., S. Zinn, D. Boarman & Allegra, C. (1990). Interaction of γ-interferon and 5-fuorouracil in the H630 human colon carcinoma cell line. *Cancer Research.* **50**:5824-5840.

Clark, A., C. Purdie, D. Harrison, R. Morris, C. Bird, M. Hooper & Wyllie, A. 1993. Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature.* **362**:849-852.

Clark, J., S. Berger, A. Mittelman & Berger, F. (1987). Thymidylate synthase gene amplification in a colon tumour resistant to fluoropyrimidine chemotherapy. *Cancer Treatment Reports.* **71**:261-265.

Conrad, A.& Ruddle, F. (1972). Regulation of thymidylate synthase activity in cultured mammalian cells. *Journal of Cell Science.* **10**:471-476.

Cory, J., J. Breland &Carter, G. (1979). Effect of 5-Fluorouracil on RNA metabolism in Novikoff hepatoma cells. *Cancer Research.* **39**:4905-4913.

Courtenay, V. & Mills, J. (1978). An in vitro colony assay for human tumorsgrown in imune-suppressed mice and treated *in vivo* with cytotoxic agents. *British Journal of Cancer.* **37**:261-268.

Corfu-A Study Group,. (1995). Phase III randomized study of two fluorouracil combinations with either interferon alfa-2a or leucovorin for advance colorectal cancer. *Journal of Clinical Oncology.* **13**:921-928.

Creasey, A., H. Smith, A. Hackett, K. Fukuyama, W. Epstein & Madin, S. (1979). Biological properties of human melanoma cells in culture. *In vitro Cell Developmental Biology.* **15**:342-350.

Danenberg, P. (1977). Thymidylate Synthase - A Target Enzyme in Cancer Chemotherapy. *Biochimica et Biophysica Acta.* **473**:73-92.

Dexter, D., J. Barbosa & Calabresi, P. (1979). N,N-Dimethylformamide-indiced Alteration of Cell Culture Characteristics and Loss of Tumorigenicity in Cultured Human Colon Carcinoma Cells. *Cancer Research.* **39**:1021-1025.

Dolnick, B.& Pink, J. (1985). Effects of 5-fluorouracil on dihydrofolate reductase mRNA from methotrexate-resistant cells. *Journal of Biological Chemistry.* **260**:3006-3010.

Domin, B., W. Mahony & Zimmerman, T. (1993). Transport of 5-Fluorouracil and uracil into human erythrocytes. *Biochemical Pharmacology.* **46**:503-510.

Drake, J., C. Allegra & Johnston, P. (1993). Immunological quantitation of thymidylate synthase-FdUMP-5,10-methlenetetrahydrofolate ternary complex with the monoclonal antibody TS 106. *Anti-cancer Drugs.* **4**:431-435.

Drake, J., V. DM, C. Allegra & Johnston, P. (1995). The effect of dose and interval between 5-fluorouracil and leucovorin on the formation of thymidylate synthase ternary complex in human cancer cells. *British Journal of Cancer.* **71**:1145-1150.

Drewinko, B., M. Romsdahl, L. Yang, M. Ahearn & Trujillo, J. (1976).

Establishment of a Human Carcinoembryonic Antigen-producing Colon Adenocarcinoma Cell Line. *Cancer Research.* **36**:467-475.

Dufour, P., F. Husseini, B. Dreyfus, H. Cure, C. Martin, G. Prevost, J. Olivier, F. Dumas, B. Duclos, R. Olivares, A. Leszler, J. Bergerat, B. Audhuy, L. Thill & Oberling, F. (1996). 5-fluorouracil versus 5-fluorouracil plus alpha-interferon as treatment of metastatic coloreactl carcinoma. A randomised study. *Annals of Oncology.* **7(6)**:575-579.

Dusenbury, C., M. Davis, T. Lawrence & Maybaum, J. (1991). Induction of megabase DNA fragments by 5-fluorodeoxyuridine in human colorectal tumour (HT-29) cells. *Molecular Pharmacology.* **39(3)**:285-289.

Eda, H., K. Fujimoto, S.-I. Watanabe, M. Ura, A. Hino, Y. Tanaka, K. Wada & Ishitsuka, H. (1993). Cytokines Induce Thymidine Phosphorylase Expression in Tumour Cells and Make Them More Suscepible To 5'deoxy-5-florouridine. *Cancer Chemotherapy and Pharmacol.oogy* **32**:333-338.

El-Assouli, S. (1985). The molecular basis for the differential sensitivity of B and T lymphocytes to growth inhibition by thymidine and 5-fluorouracil. *Leukemia Research.* **9**:391-398.

Elias, L. & Crissman, H. (1988). Interferon effects upon adenocarcinoma 38 and HL-60 cell lines: Antiproliferative responses and synergistic interactions with halogenated pyrimidine anti-metabolites. *Cancer Res. earch***48**:4868-73.

Engel, L., N. Young, T. Tralka, M. Lippman, S. O'Brien & Joyce, J. (1978). Establishment and characterisation of three new continuous cell lines derived from human breast carcinomas. *Cancer Research.* **38**:3352-3364.

Eva, A., K. Robbins, P. Anderson, A. Srinivasan, S. Tronick, P. Reddy, N. Ellmore, A. Galen, J. Lautenberger, T. Papas, E. Westin, F. Wong-Staal, R. Gallo & Aaronson, A. 1982. Cellular genes analogous to retroviral *onc* genes are transcribed in human tumour cells. *Nature.* **295**:116-119.

Evans, R., J. Laskin & Hakala, M. (1980). Assessment of growth-limiting events caused by 5-fluorouracil in mouse cells and in human cells. *Cancer Research.* **40**:4113-4122.

Evans, R., J. laskin & Hakala, M. (1981). Effect of excess folates and deoxyinosine on the activity and site of action of 5-fluorouracil. *Cancer Research.* **41**:3288-3295.

Fearon, E., K. Cho, J. Nigro, S. Kern, J. Simons & Ruppert., J. (1990). Identification of a chromosome 18q gene that is altered in colorectal cancers. *Science.* **247**:49-56.

Felgner, P., T. Gadek, M. Holm, R. Roman, H. Chan, M. Wenz, J. Northrop, G. Ringhold & Danielsen, M. (1987). Lipofection: A highly efficient, lipid mediated DNA-transfection prcedure. *Proceedings of the National Academy of Science* **84**:7413.

Findlay, M., A. Hill, D. Cunningham, A. Norman, M. Nicolson, H. Ford, J. Husband, C. Evans & Carter, R. (1994). Protracted venous infusion of 5-fluorouracil and interferon-α in advanced and refractory colorectal cancer. *Annals of Oncology.* **5**:239-243.

Finnis, C., N. Dodsworth, C. Pollitt, G. Carr & Sleep, D. (1993). Thymidine phosphorylase activity of platelet-derived endothelial cell growth factor is responsible for endothelial cell mitogenicity. *European Journal of Biochemistry.* **212**:201-210.

Fogh, J. & Trempe, G. (1975). New human tumor cell lines. New York and London: Plenum Press.

Fox, S., K. Engels, M. Comley, R. Whitehouse, H. Turley, K. Gatter & Harris, A. (1997). Relationship of elevated tumour thymidine phosphoryalse in node-positive breast carcinomas to the effects of adjuvant CMF. *Annals of Oncology.* **8**:271-275.

Fox, S., K. Gatter, R. Bicknell, J. Going, P. Stanton, T. Cooke & Harris, A. (1993.) Relationship of endothelial cell proliferation to tumour vascularity in human breast cancer. *Cancer Research.* **53**:4161-4163.

Fox, S., A. Moghaddam, M. Westwood, H. Turley, R. Bicknell, K. Gatter & A. Harris. (1995). Platelet-derived endothelial cell growth factor/thymidine phosphorylase expression in normal tissues: An immunohistochemical study. *Journal of Pathology.* **176**:183-190.

Fox, S., M. Westwood, A. Moghaddam, M. Comley, H. Turley, R. Whitehouse, R. Bicknell, K. Gatter & Harris, A. (1996). The angiogenic factor platelet-derived endothelial cell growth factor/thymidine phosphorylase is up-regulated in breast cancer epithelium and endothelium. *British Journal of Cancer.* **73**:275-280.

Freidkin, M. & Roberts, A. (1953). The enzymatic sythesis of nucleotides. 245-256.

Freshney, R. (1987). Culture of animal cells: A manual of basic technique (2nd edition ed.). Wiley-Liss.

Fujiwaki, R., K. Hata, K. Iida, M. Koike & Miyazaki, K. (1998). Immunohistochemical expression of thymidine phosphorylase in human endometrial cancer. *Gynaecological Oncoloy.* **68**:247-252.

Furukawa, T., A. Yoshimura, S. Tomoyuki, H. Misako & Akiyama, S. (1992). Angiogenic factor. *Nature.* **356**:668.

Gallo, R., S. Perry & Breitman, T. (1967). The enzymatic mechanisms for deoxythymidine synthesis in human leukocytes. Substrate inhibition by thymine and activation by phosphate or arsenate. *Journal of Biological Chemistry.* **242**:5059-5068.

Gan, T., L. Hallam, G. Pilkington & Van der Weyden, M. (1981). A Rapid and Simple Radiometric Assay for Thymidine Phosphorylase of Human Peripheral Blood cells. *Clinica Chimica Acta.* **116**:231-236.

Gazdur, A., D. Carney, E. Russel, H. Smis, S. Bylin, J. Bunn, G. Guccion & Minna, . 1980. Establishment of continuous clonable culture of small cell carcinoma of the lung which have amino precursor uptake and decarboxylation cell properties. *Cancer Research.* **40**:3502-3507.

Giard, D., S. Aaronson, G. Todaro, J. Kersey, H. Dosik & Parks, W. (1973). *In vitro* cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *Journal of the National Cancer Institute.* **51**:1417-1423.

Goulian, M., B. Bleile, L. Dickey, R. Grafstrom, H. Ingraham, S. Neynaber, M. Peterson & Tseng, B. (1986). Mechanism of thymineless death. *Advances in Experimental Medical Biology.* **195**:89-95.

Grafstrom, R., B. Tseng & Goulian, M. (1978). The incorporation of uracil into animal cell DNA *in vitro. Cell.* **15**:131-140.

Graham, F. & Van Der Eb, A. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology.* **52**:456-67.

Greenhalgh, D.& Parish, J. (1990). Effect of 5-Fluorouracil combination therapy on RNA processing in human colonic carcinoma cells. *British Journal of Cancer.* **61**:415-419.

Grem, J.& Fischer, P. (1989). Enhancement of 5-fluorouracil's anticancer activity by dipyridamole. *Pharmacology and Therapeutics* **40**:349-371.

Hamburger, A. & Salmon, S. (1977). Primary bioassay of human tumour stem cells. *Science.* **197**:461-463.

Hamilton, T., R. Young, K. McKoy, J. Green, E. CHu, C. Whabg-Peng & Rogan, A. (1983). Characterisation of a human ovarian carcinm cell line (NIH:OVCAR-3) with androgen and estrogen receptors. *Cancer Research.* **43**:5379-5389.

Hande, K. & Chabner, B. (1978). Pyrimidine nucleoside monophosphate kinase from human leukemic blast cells. *Cancer Research.* **38(3)**:579-585.

Haraguchi, M., T. Furukawa, T. Sumizawa & Akiyama, S. (1993). Sensitivity of Human KB Cells Expressing Platelet-derived Endothelial Cell Growth Factor to Pyrimidine Antimetabolites. *Cancer Research.* **53**:5680-5682.

Hata, K., H. Nagami, K. Iida, K. Miyazaki & Collins, W. (1998). Expression of thymidine phosphorylase in malignant ovarian tumors: correlation with microvessel density and an ultrasound-derived index of angiogenesis. *Ultrasound Obstetrics and Gynaecology.* **12**:201-206.

Heggie, G., J. Sommadossi & Cross, D. (1987). Clinical pharmacokinetics of 5-fluorouracil and it's metabolites in plasma, urine and bile. *Cancer Research.* **1987**:2203-2206.

Heidelberger, C., N. Chaudhauri & Danenberg, P. (1957). Fluorinated pyrimidines: a new class of tumour inhibitory compounds. *Nature.* **179**:663-666.

Heldin, N., K. Usuki, J. Bergh, B. Westermark & Heldin, (1993). Differential Expression of Platelet-derived Endothelial Cell growth Factor/Thymidine Phosphorylase in Human Lung Carcinoma Cell Lines. *British. J.ournal of Cancer.* **68**:708-711.

Herrick, D.& Kufe, D (1984). Lethality associated with incorporation of 5-fluorouracil into preribosomal RNA. *Molecular Pharmacology*. **26(1)**:135-140.

Hill, B. (1983). Use of continuous human tumour cell lines to evaluate drugs by clonogenic assays. New York: Academic Press.

Hill, M., A. Normann, D. Cunningham, M. Findlay, V. Nicolson, A. Hill, A. Iveson, C. Evans, J. Joffe & Nicolson, M. (1995). Royal Marsden phase III trial of fluorouracil with or without interferon α-2b in advanced colorectal cancer. *Journal of Clinical Oncology*. **13**:1297-1302.

Houghton, J., D. Adkins, A. Rahman & Houghton, P. (1991). Interaction between 5-fluorouracil, [6RS]Leucovorin, and recombinant human interferon-α2a in cultured colon adenocarcinoma cells. *Cancer Communications*. **3**:225-231.

Houghton, J. & Houghton, P. (1983). Elucidation of pathways of 5-fluorouracil metabolism in xenografts of human colorectal adenocarcinoma. *European Journal of Clinical Oncology*. **19(6)**:807-815.

Houghton, J., J. Maroda SJ, J. Phillips & Houghton, P. (1981). Biochemical Determinants of Responsiveness to 5-Fluorouracil and its Derivatives in Xenografts of Human Colorectal Adenocarcinomas in Mice. *Cancer Research*. **44**:144-149.

Houghton, J., C. Morton, D. Adkins & Rahman, A. (1993). Locus of the interaction among 5-fluorouracil, leucovorin and interferon-α2a in colon carcinoma cells. *Cancer Research*. **53**:4243-4250.

Houghton, J. A. & . Houghton, P. J. (1980). 5-Fluorouracil in combination with hypoxanthine and allopurinol: toxicity and metabolism in xenografts of human colonic carcinomas in mice. *Biochemical Pharmacology*. **29**:2077-2081.

Houghton, J., L. William, S. de Graaf, P. Cheshire, J. Rodman, D. Maneval, I. Wainer, P. Jadaud & Houghton, P. (1990). Relationship between dose rate of [6*RS*]Leucovorin administration, plasma concentrations of the reduced folates and pools of 5,10-methylenetetrahydrofolates and tetrahydrofolates in human colon adenocarcinoma xenografts. *Cancer Research*. **50**:3493-3502.

Huberman, M., E. Clay & M. Atkins. (1991). Phase II trial of 5-fluorouracil (5-FU) and recombinant interferon-alpha-2a (IFN) in advanced colorectal cancer. *Proceedings of the Americanc Society for Clinical Oncology.* **10**: 153 (Abstract 478)

Igarashi, M., D. Dhar, H. Kubota, A. Yamamoto, O. El-Assal & Nagasue, N. (1998). The prognostic significance of microvessel density and thymidine phosphorylase expression in squamous cell acrcinoma of the esophagus. *Cancer.* **82(7)**:1225-1232.

Iltzsch, M., M. el Kouni & Cha, S. (1985). Kinetic studies of thymidine phosphorylase from mouse liver. *Biochemistry.* **24**:6799-6807.

Inada, T., A. Ichikawa, T. Kubota, Y. Ogata, A. Moossa & Hoffman, R. (1997). 5-FU-induced apoptosis correlates with efficacy against human gastric and colon cancer xenografts in nude mice. *Anticancer Research.* **17**:1965-1972.

Ingraham, H., B. Tseng & Goulian, M. (1980). Mechanism for exclusion of 5-fluorouracil from DNA. *Cancer Research.* **40**:998-1001.

Ishikawa, F., K. Miyazono, U. Hellman, H. Drexler, C. Wernstedt, K. Hagiwara, K. Usuki, F. Takaku, W. Risau & Heldin, C. (1989). Identification of angiogenic activity and the cloning and expression of platelet-derived endothelial cell growth factor. *Nature.* **338**:557-562.

John, W., J. Neefe, J. Macdonald, J. J. Cantrell & Smith, M. (1993). 5-Fuorouracil and interferon-alpha-2a in advanced colorectal cancer. *Cancer.* **72(11)**:3191-3195.

Johnson, L. (1984). Expression of dihydrofolate reductase and thymidylate synthase in mammalian cells. New York: Academic Press.

Johnston, P., J. Drake, J. Trepel & Allegra, C. (1992). Immunological Quantitation of Thymidylate Synthase Using the Monoclonal Antibody TS 106 in 5-Fluorouracil-sensitive and -Resistant Human Cancer Cell Lines. *Cancer Research.* **52**:4306-4312.

Johnston, P., E. Fisher, H. Rockette, B. Fisher, N. Wolmark, J. drake, B. Chabner & Allegra, C. (1994). The Role of Thymidylate Synthase Expression in Prognosis and Outcame of Aduvant Chemotheraoy in Patients With Rectal Cancer. *Journal of Clinical Oncology.* **12**:2640-2647.

Johnston, P., H.-J. Lenz, C. Leichman, K. Danenberg & Leichman, L. (1995). Thymidylate synthase gene and protein expression correlate and are associated with response to 5-FU in human colorectal and gastric tumours. *Cancer Res.earch* **55**:1407-1412.

Johnston, P., C. Liang, S. Henry, B. Chabner & Allegra, C. (1991). The production and characterisation of monclonal antibodies that localize human thymidylate synthase in the cytoplasm of human cells and tissues. *Cancer Research.* **51**:6668-6677.

Kastan, M., O. Onyekwere, D. Sidransky, B. Vogelstein & Craig, R. (1991). Participation of p53 protein in the cellular response to DNA damage. *Cancer Research.* **51**:6304-6311.

Kato, Y., S. Matsukawa, R. Muraoka & Tanigawa, N. (1997). Enhancement of drug sensitivity and a bystander effect in PC-9 cells transfected with a platelet-derived endothelial cell growth factor thymidine phosphorylase cDNA. *British Journal of Cancer.* **75(4)**:506-511.

Kawai, S. & Nishizawa, M. (1984). New procedure for DNA transfection with polycation and dimethyl sulfoxide. *Molecular Cell Biology.* **4(6)**:1172-1174.

Kemeny, N., D. Younes, A. Seietr, K. (1990). Interferon alpha-2a and 5-fluorouracil fro advanced colorectal carcinoma. Assessment of toxicity. *Cancer (*1990) **66**: 2470-2475

Keyomarsi, K. & R. Moran. (1986). Folinic acid augmentation of the effects of fluoropyrimidines on murine and human leukemic cells. *Cancer Research.* **46**:5229-5235.

Kingston, R. (1997). Introduction of DNA into mammalian cells. John Wiley & Sons Inc.

Kohne, C., H. Wilke, H. Hecker, P. Schoffski, C. Kaufer, H. Rauschecker, R. Andreesen, U. Ohl, H. Lange & U. Klaasen. (1995). Interferon-alpha does not improve the antineoplastic efficacy of high-dose infusional 5-fluorouracil plus folinic acid in advanced colorectal cancer. First results of a randomized multicentre study by the Association of Medical Oncology of the German Cancer Society. *Annals of Oncology.* **146(5)**:461-466.

Koukourakis, M., A. Giatromanolaki, K. O'Byrne, M. Comley, R. Whitehouse, D. Talbot, K. Gater & A. Harris. (1997). Platelet-derived endothelial cell growth factor expression correlates with tumour angiogenesis and prognosis in non-small-cell lung cancer. *British Journal of Cancer.* **75**:477-481.

Kouni, M. e. & Kouni, M. e. (1993). Differences in Activities and Substrate Specificity of Human and Murine Pyrimidine Nucleoside Phosphorylases: Implications for Chemotherapy with 5-Flouropyrimidines. *Cancer Research.* **53**:3687-3693.

Krenitsky, T. (1967). Pentosyl transfer mechanisms of the mammalian nucleoside phosphorylases. *Journal of Biological Chemistry.* **243**:2871-2875.

Krenitsky, T. (1967). Purine nucleoside phosphorylase: kinetics, mechanism and specificity. *Molecular Pharmacology.* **3**:526-536.

Kreuser, E., N. Streitand T. Kuchler. (1995). A multicenter randomised trial with the assessment of the quality of life in patients with metastatic colorectal carcinoma given either folinic acid or interferon-α-2b as a modulator of 5-fluorouracil. *Proceedings of the American Society for Clinical Oncology.* **14**:202.

Kufe, D.& Major, P. (1981). 5-fluorouracil incorporation into human breast carcinoma RNA correlates with cytotoxicity. *Journal of. Biological. Chem.istry* **256**:9802.

Laemmli, U. (1970). Cleavage of structure points during the assembly of the head of bacteriophase. *Nature.* **277**:680-5.

Lane, D. (1992). p53 guardian of the genome. *Nature.* **258**:15-16.

Laskin, J., R. Evans, H. Slocum, D. Burke & Hakala, M. (1979). Basis for natural variation in sensitivity to 5-fluorouracil in mouse and human cells in culture. *Cancer Research.* **39**:383-390.

Lenz, H., C. Leichman, P. Danenberg, H. Silberman, T. Horokoshi, M. Kiyabu, K. Danenberg, C. Spears, L. Laine, M. Fuerstand L. Leichman. (1993). Thymidylate synthase (TS) gene expression predicts response of primary gastric cancer (GC) to 5-fluorouracil (5-FU)-Leucovorin (LV)-Cisplatin (DDP). *Proceedings of the ASCO.* **12**:199.

Leventis, R.& Silvius, J. (1990). Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles. *Biochimica et. Biophysica. Acta.* **1023**:124-132.

Levine, E. (1972). Mycoplasma contamination of animal cell cultures: a simple rapid detection method. *Experimental Cell Research.* **74**:99-109.

Lonn, U.and S. Lonn. (1984). Interaction between 5-Fluorouraicil and DNA of human colon adenocarcinomas. *Cancer Research.* **44**:3414-3418.

Lonn, U.& Lonn, S. (1986). DNA lesions in human neoplastic cells and cytotoxicity of 5-Fluoropyimidines. *Cancer Research.* **46**:3866-3870.

Lonn, U.& Lonn, S. (1988). Increased levels of DNA lesions induced by leucovorin-5-fluoropyrimidine in human colon adenocarcinoma. *Cancer Research.* **48(15)**:4153-4157.

Lowe, S., H. Ruley, T. Jacks & Housman, D. (1993). p53-Dependent Apoptosis Modulates the Cytotoxicity of Anticancer Agents. *Cell.* **74**:957-967.

Luccioni, C., J. Beaumatin, V. Bardot & Lefrancois, D. (1994). Pyrimidine Nucleotide Metabolism in Human Colon Carcinomas: Comparison of Normal Tissues, Primary Tomours and Xenografts. *International. Journal of. Cancer.* **58**:517-522

Machover, D., E. Goldschmidt & Chollet, P. (1986). Treatment of advanced colorectal and gastric adenocarcinomas with 5-fluorouracil and high-dose folinic acid. *Journal of. Clinical. Oncol.ogy* **4**:685.

Madoc-Jones, H. & Bruce, W. (1968). On the mechanism of the lethal action of 5-fluorouracil on mouse L cells. *Cancer Research.* **28**:1976-1981.

Maeda, K., Y. Chung, Y. Ogawa, S. Takatsuka, S. KAng, M. Ogawa, S. T, N. Onoda, Y. Kato & Sowa, M. (1996). Thymidine phosphorylase/platelet-derived endothelial cell growth factor expression associated with hepatic metastasis in gastric carcinoma. *British Journal of Cancer.* **73:**884-888.

Major, D., E. Egan, D. Herrick & Kufe, D. (1982). 5-Fluorouracul incorporation in DNA of human breast cancer cells. *Cancer Research.* **42**:3005-3009.

Martin, D., R. Nayak, R. Sawyer, R. Stolfi, C. Young, T. Woodcock & S. S. (1978). Enhancement of 5-fluorouracil chemotherapy with emphasis on the use of excess thymidine. *Cancer Bulletin.* **30**:219-224.

Martoni, A., L. Bidin, C. Zamagni, A. Cricca & Pannuti, F. (1995). Failure to Increase 5-Fluorouracil Activity with Interferon-a2a in the Treatment of Advanced Colorectal Cancer. *European Journal of Cancer.* **31A**:127.

Mauro, D., J. De Reil, R. Tallarida & Sirover. M. (1993). Mechanisms of excision of 5-fluorouracil by uracil DNA glycosylase in normal human cells. *Molecular Pharmacology.* **43**:854-859.

McCutchan, J. & Pagano, J. (1968). Enhancement of the infectivity of Simian Virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran. *Journal of the National Cancer Institute.* **41**:351-357.

McIlwrath, A., P. Vasey, G. Ross & Brown, R. (1994). Cell cycle arrests and radiosensitivity of human tumor cell lines: dependance on WT p53 for radiosensitivity. *Cancer Research.* **54**:3718-3722.

Metzger, R., K. Danenberg, C. Leichman, D. Salonga, E. Schwartz, S. Wadler, H. Lenz, S. Groshen, S. Leichman & Danenberg, P. (1998). High basal level gene expression of thymidine phosphorylase (platelet-derived endothelial cell growth factor) in colorectal tumors is associated with non-response to 5-fluorouracil. *Clinical Cancer Research.* **4**:2371-2376.

Miwa, M., A. Cookand H. Ishisuka. (1986). Enzymatic cleavage of carious fluorinated pyrimidine nucleosides to 5-fluorouracil and their antiproliferative activities in human and murine tumor cells. *Chemistry and Pharmacy Bulletin.* **34**:4225-4232.

Miyadera, K., T. Sumizawa, M. Haraguchi, H. Yoshida, W. Konstanty, Y. Yamada & Akiyama, S. (1995). Role of thymidine phosporylase activity in the angiogenic effect of platelet-derived endothelial cell growth factor/thymidine phosphorylase. *Cancer Research.* **55**:1687-1690.

Miyazono, K. & Heldin, C. (1989). High-yield purification of PD-ECGF: structural characterisation and establishment of a specific antiserum. *Biochemistry.* 28:1704-1710.

Miyazono, K., T. Okabe, A. Urabe, F. Takakuand C. Heldin. 1987. Purification and properties of an endothelial cell growth factor from human platelets. *The Journal of Biological Chemistry.* **262**:4098-4103.

Moghaddam, A. & Bicknell., R (1992) Expression of Platelet-Derived Endothelial Cell Growth Factor in Escherichia coli and Confirmation of its Thymidine Phosphorylase Activity. *Biochemistry.* **31**:12141-12146.

Moghaddam, A., Z. Hua-Tang, T. Fan, D. HU, V. Lees, H. Turley, S. Fox, K. Gatter, A. Harris & R. Bicknell. (1995). Thymidine phosphorylase is angiogenic and promotes tumour growth. *Proceedings of the. National. Academy of. Science. USA.* **92**:998-1002.

Moran, R., C. Spears & Heidelberger, C. (1979). Biochemical determinants of tumour sensitivity to -5-fluorouracil: Ultrasensitive methods for the determination of 5-fluoro-2'-deoxyuridylate, 2'-deoxyuridylate and thymidylate synthase. *Proceedings of the National Academy of Science, USA.* **76**:1456-1460.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods.* **65**:55-63.

Murgo, A., J. Fried, D. Burchenal, K. Vale, A. Strife, T. Woodcock, C. Young & Clarkson, B. (1980). Effects of thymidine and thymidine plus 5-fluorouracil on the growth kinetics of a human lymphoid cell line. *Cancer Research.* **40(5)**:1543-1549.

Myers, C., R. Young & Chabner, B. (1975). Biochemical determinants of 5-fluorouracil response in vivo: the role of deoxyuridylate pool expansion. *Journal of Clinical Investigations.* **56**:1231-1238.

Nadal, J., C. van Greoningen, H. Pinedo & Peters, G. (1988). In vivo potentiation of 5-fluorouraicl by leucovorin in murine colon carcinoma. *Biomedicine & Pharmacotherapy.* **42**:387-393.

Nadal, J., C. van Groeningen, H. Pinedoand Peters, G. (1989). Scedule-dependancy of *in vivo* modulation of 5-fluorouracil by leucovorin and uridine in murine colon carcinoma. *Investigational New Drugs.* **7**:163-172.

Naguib, F., A. El Kouni & Cha, S. (1985). Enzymes of uracil catabolism in normal and neoplastic human tissues. *Cancer Research.* **45**:5405-5412.

Nayak, R. (1992). Thymidine inhibits the incorporation of 5-Fluoro-2'-Deoxyuridine to DNA of Mouse Mammary Tumour. *Biochemical and Biophysical Research Communications.* **184**:467-470.

Neumann, E., M. Schaefer-Ridder, Y. Wang & Hofschneider, P. (1982). Gene transfer into mouse lyoma cells by elctroporation in high electric fields. *EMBO Journal.* **1(7)**:841-845.

O'Brien, S., M. Kirkland & Goldman, (1994). European School of Oncology Task Force Papers: Gene Therapy-a Future in Cancer Management? Antisense Therapy for Malignant Disease. *European Journal of Cancer.* **30A**:1160-1164.

O'Brien, T., D. Cranston, S. Fuggle, R. Bicknell & Harris, A. (1995). Different angiogenic pathways characterise superficial and invasive bladder cancer. *Cancer Research.* **55**:510-513.

Oltvai, Z., C. Miliman & Korsmeyer, K. (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell.* **74**:609-619.

Patterson, A., H. Zhang, A. Moghaddam, R. Bicknell, D. Talbot, I. Stratford & Harris, A. (1995). Increased Sensitivity to the Pro-Drug 5'deoxy-5-fluorourdine and Modulation of 5'deoxy-5-fluorourdine sensitivity in MCF-7 Cells Transfected with Thymidine Phosphorylase. *British Journal of Cancer.* **72**:669-675.

Pauly, J., N. Paolini, R. Ebarb & Germain, M. (1978). Elevate thymidine phosphorylase activity in the plasma and ascitic fluids of tumour-bearing animals. *Proceedings of the Society for Experimental Biology and Medicine.* **157**:262-267.

Pauly, J., M. Schuller, A. Zelcer, T. Kirss, S. Gore & Germain, M.(1977). Identification and Comparative Analysis of Thymidine Phosphorylase in the Plasma of Healthy Subjects and Cancer patients: Brief communication. *Journal; of the National Cancer Inst.itute* **58**:1587-1590.

Pazdur, R., J. Ajani, Y. Patt, J. Gomez, B. Bready & Levin, B. (1993). Phase II evaluation of recombinant alpha-2a-interferon and continuous infusion fluorouracil in previously untreated metastatic colorectal carcinomas. *Cancer.* **71(4)**:1214-1218.

Pazdur, R., J. Ajani, Y. Patt, R. Winn, D. Jackson, B. Shepard, R. DuBrow, L. Campos, M. Quaraishi, J. Faintuch, J. Abbrzzese, J. Gutterman & B. Levin. (1990). Phase II study of fluorouracil and recombinant interferon alfa-2a in previously untreated advanced colorectal carcinoma. *Journal of Clinical Oncology.* **8**:2027-2031.

Peebles, P., T. Trisch & Papageorge, A. (1978). Isolation of four unusual paediatric solid tumor cell lines. *Pediatric Research.* **12**:485.

Peidbois, P., M. Buyse & Rustum, M. (1992). Modulation of fluorouracil by leucovorin in patients with advanced colorectal cancer: evidence in terns of response. *Journal; of. Clinical. Oncology.* **10**:896.

Pestalozzi, B., C. McGinn, T. Kinsella, J. Drake, C. Allegra & Johnston, P. (1995). Increased thymidylate synthase protein levels are principally assocaited with proliferation but not cell cycle phase in asynchronous human cancer cells. *British Journal of Cancer.* **71**:1151-1157.

Peters, G., E. Laurensse, A. Leyva, J. Lankelma & Pinedo, H. (1986). Sensitivity of human, murine and rat cells to 5-fluorouracil and 5'-deoxy-5-fluorouridine in relation to drug-metabolising enzymes. *Cancer Research.* **46**:20-28.

Pinedo, H. & Peters, G. (1988). Fluorouracil: Biochemistry and Pharmacology. *Journal of Clinical Oncology.* **6**:1653-1664.

Piper, A. & Fox, R.(1982). Biochemical Basis for the Differential Senstitivity if Human T- and B-Lymphocyte Lines to 5-Fluorouracil. *Cancer Research.* **42**:3753-3760.

Plumb, J., R. Milroy & Kaye, S. (1989). Effects of pH Dependence of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide-Formazan Absorption on Chemosensitivity Determined by a Novel Tetrazolium-based Assay. *Cancer Research.* **49**:4435-440.

Poon, M., O'Connell. M.J. & Weiand. HS. (1991). Biochemical modulation of fluorouracil with Leucovorin. confirmatory evidence of improved therapeutic efficacy in advanced colorectal cancer. *J.ournal of Clinical Oncology* **9**:1967-1972.

Poon, M., O'Connell, M & Moertel, C. (1989). Biochemical modulation of fluorouracil: evidence of significant improvement of survival and quality of life in patients with advanced colorectal carcinoma. *Journal of Clinical.Oncology* **7**:1407.

Priest, D., C. Alford, K. Batson & Doig, M. (1980). A centrifugal column assay for thymidylate synthetase using the active site titrant 5-fluoro-2-deoxyuridylate. *Analytical Biochemistry*. **103**:51-4.

Puck, T. & Marcus, P. (1955). A rapid method of viable cell titration and colony production with HeLa cells in tissue culture: the use of x-irradiated cells to supply conditioning factors. *Proceedings of the National Academy of Science*. **41**:432-437.

Radparvar, S., P. Houghton & Houghton, J. (1989). Effect of polyglutamation of 5,10-methylenetetrahydrofolate on the binding of 5-flouro-2'-deoxyuridylate to thymidylate synthase purified from a human colon adenocarcinoma xenograft. *Biochemical Pharmacology*. **38**:335-342.

Reichard, P., O. Skold & , Klein, G. (1959). Possible enzymatic mechanisms for the development of resistance against Fluorouracil in ascites tumours. *Nature*. **183**:939-941.

Relf, M., S. LeJeune, P. Scott, A. Scott, S. Fox, K. Smith, R. Leek, A. Moghaddam, R. Whitehouse, R. Bicknell & Harris, A. (1997). Expression of the angiogenic factors Vascular Endothelial Cell Growth Factor, Acidic and Basic Fibroblast Growth Factor, Tumour Growth Factor β-1, Platelet derived Endothelial Cell Growth Factor, Placenta Growth Factor, and Pleitrophin in human primary breast cancer and its relation to angiogenesis. *Cancer Research*. **57**:963-969.

Rennie, J. & R. Rusting. (1996). Making headway against cancer. *Scientific American*. 28-30.

Reynolds, K., F. farzaneh, W. Collins, S. Campbell, T. Bourne, F. Lawton, A. Moghaddam, A. Harrisand R. Bicknell. (1994). Association of Ovarian Malignancy with Expression of Platelet-Derived Endothelial Cell Growth Factor. *Journal of the National Cancer Institute*. **86**:1234-1238.

Romanini, A., J. Lin, D. Neidzwiecki, M. Bunni, D. Priest & Bertino, J. (1991). Role of folylpolyglutamates in biochemical modulation of fluoropyrimidines by leucovorin. *Cancer Research*. **51**:789-793.

Rubio, D., J. Jimeno & Camps, C. (1992). Treatment of advanced colorectal cancer with recombinant interferon alpha and fluorouracil: activity in liver metastasis. *Cancer Investigations*. **10**:259-264.

Rutman, R., A. Cantarrow & Paschkis, K. (1954). Studies on 2'-acetylaminofluorene carcinogenesis:III. The utilisation of uracil-2-C^{14} by preneoplastic rat liver. *Cancer Research.* **14**:119.

Saltz, L.& Kelsen, D. (1997). Adjuvant treatment of colorectal cancer. *Annual Review of Medicine.* **48**:191-202.

Santelli, G. & Valeriote, F. (1978.) *In vivo* enhancement of 5-fluorouracil cytotoxicity to AKR leukemia cells by thymidine in mice. *Journal of the National Cancer Institute.* **61**:843-847.

Santi, D., C. McHendry & Sommer, H. (1974). Mechanism of Interaction of Thymidylate Synthetase with 5-Fluorodeoxyuridylate. *Biochemistry.* **13**:471-481.

Sawyer, R., R. Stolfi, D. Martin & Speigelman, S. (1984). Incorporation of 5-fluorouracil into murine bone marrow DNA *in vivo*. *Cancer Research.* **44:**1847-1851.

Schuetz, J. & Diasio, R. (1985). The effect of 5-fluorouracil on DNA chain elongation in intact bone marrow cells. *Biochemical and Biophysical Research Communications.* **133**:361-367.

Schwartz, E., N. Baptiste, S. Megati, S. Wadler & Otter, B. (1995). 5-Ethoxy-2'-deoxyuridine, a Novel Substrate for Thymidine Phosphorylase, Potentiates the Antitumour Activity of 5-Fluorouracil When Used in Combination with Interferon, an Inducer of Thymidine Phosphorylase Expression. *Cancer Research.* **55**:3543-3550.

Schwartz, E., N. Baptiste, C. O'connor, S. Wadler & Otter, B. (1994). Potentiation of the Antitumour Activity of 5-Flourouracil in Colon Carcinoma cells by the Combination of Interferon and Deoxyribonucleotides Results from Complementary Effects on Thymidine Phosphorylase. *Cancer Research* **54**: 1472-1478.

Schwartz, E., N. Baptiste, S. Wadler and Makower, D. (1995). Thymidine phosphorylase mediates the sensitivity of human colon carcinoma cells to 5-Fluorouracil. *Journal of. Biol.ogical Chemistry.* **270**:19073-19077.

Schwartz, E., M. Hoffman, C. O'Connor & S. Wadler. (1992). Stimulation of 5-fluorouracil metabolic activation by Interferon-α in human colon carcinoma cells. *Biochemical and Biophysical Research Communications.* **182**:1232-1239.

Schwartz, M. (1971). Thymidine Phosphorylase from *Escherichia coli. European Journal of Biochemistry.* **21**:191-198.

Schwartz, P., J. Dunigan, J. Marsh & Handschumacker, R. (1980). Allopurinol modification of the toxicity and antitumour activity of 5-fluorouracil. *Cancer Research.* **40(6)**:1885-1889.

Schwartz, P. & Handschumacher, R. (1979). Selective antagonism of 5-fluorouracil cytotoxicity by 4-hydropyrazolopyrimidine (allopurinol) *in vitro. Cancer Research.* **39**:3095-3101.

Shah, V., K. Midha, S. Dighe, I. McGilveray, J. Skelly, A. Yacobi, T. Layoff, C. Viswanathan, C. Cook, R. McDowall, K. Pittman & S. Spector. 1992. Analytical methods validation: bioavailability,bioequivalence, and pharmacokinetics studies. *Journal of Pharmaceutical Sciences.* **81**:309-3112.

Siegel, D., N. Gibson, P. Preusch & D. Ross. (1990). Metabolism of Mitomycin C by DT-Diaphorase: Role in Mitomycin C-induced NA Damage and Cytotoxity in Human Colon Carcinoma Cells. *Cancer Research.* **50**:7483-7489.

Sotos, G., L. Groganand & Allegra., C (1994). Preclinical and clinical aspects of biomodulation of 5-fluorouracil. *Cancer Treatment Review.* **20**:11-49.

1Soule, H. (1973). A human cell line form a pleural effusion derived from a breast carcinoma. *Journal of the National Cancer Institute.* **51**:1409-1416.

Southern, P.& P. Berg. (1982). Transformation of mammalian cells to antibiotic resistance with a bacterial gene under the control of the SV40 early region promoter. *Journal of Molecular Applied Genetics.* **1**:327-341.

Spears, C., B. Gustavsson, M. Mitchell, D. Spicer, M. Berne, L. Bernstein & Danenberg, P. 1984. Thymidylate synthetase inhibition in malignant tumors and normal liver of patients given intravenous 5-Fluorouracil. *Cancer Research.* **44**:4144-4150.

Spears, C., A. Shahinian, R. Moran, C. Heidelberger & T. Corbett. (1982). *In vivo* kinetics of thymidylate synthetase inhibition in 5-fluorouracil-sensitive and -resistant murine colon adenocarcinoma. *Cancer Research.* **42**:450-456.

Speigelman, S., R. Nayak, R. Sawyer, R. Stolfi & Martin, D. (1980). Potentialtion of the anti-tumour activity of 5-FU by thymiidne and its correlation with the formation of (5FU) RNA. *American Cancer Society.* **45**:1129-1134.

Speigelman, S., R. Sawyer, R. Nayak, E. Ritzi, R. Stolfi & Martin, D. (1980). Improving the anti-tumour activity of 5-fluorouracil by increasing its incorporation into RNA via metabolic modulation. *Proceedinds of the National Academy of Science.* **77**:4966-4970.

Sri-Pathmanathan, R., J. Plumband & Fearon, K. (1994). Clofazine alters the energy metabolism and inhibits the growth rate of a human lung-cancer cell line *in vitro* and *in vivo. International Journal of Cancer.* **56**:900-905.

Sumizawa, T., T. Furukawa, M. Haraguchi, A. Yoshimura, A. Takeyasu, M. Ishizawa, Y. Yamada & Akiyama, S.I.. (1993). Thymidine Phosphorylase Activity Associated with Platelet-Derived Endothelial Cell Growth Factor. *J.ournal of Biochemistry.* **114**:9-14.

Swain, S., M. Lippman, E. Egan, J. Drake, S. Steinbergand C. Allegra. 1989. Fluorouracil and high dose leucovorin in previously treated patients with with metastatic breast cancer. *Journal of Clinical Oncology.* 7:890-896.

Takebayashi, Y., K. Yamada, T. Sumizawa, K. Miyadera, T. Aikou & Akiyama, S. (1995). Clinicopathological significance of an angiogenic enzyme, thymidine phosphorylase (dThdPase), in colorectal carcinoma. *Proceedings of the American Association fot Cancer Research.* **36**:90, Abstract 535.

Tevaearai, H., P. Laurent, L. Suardet, J. Eliason, J.-C. Givel & Odartchenko, N. (1992). Interactions of Interferon-α2 a with 5'-Deoxy-5-fluorouridine in colorectal cancer cells *in vitro. European Journal of Cancer.* **28**:368-372.

Toi, M., S. Hoshina, T. Taniguchi, Y. Yamamoto, H. Ishitsuka & Tominaga, T. (1995). Expression of platelet-derived endothelial cell growth factor/thymidine phosphorylase in human breast cancer. *Int.ernational J.ournal of Cancer.* **64**:79-82.

Umeda, M. & Heidelberger, C. (1968). Comparative studies of fluorinated pyrimidines with various cell lines. *Cancer Research.* **28**:2529-2538.

Usuki, K., L. Norberg, E. Larsson, K. Miyazono, U. Hellman, C. Wernstedt, K. Rubin & Heldin, C. (1990). Localization of platelet-derived endothelial cell growth factor in human placenta and purification of an alternatively processed form. *Cell Regulation.* **1**:577-596.

Van Hoff, D. (1991). *In vitro* data supporting interferon plus cytotoxic agent combinations. *Seminars in Oncology.* **18**:58-61.

Vogelstein, B., E. Fearon, S. Hamilton, S. Kern, A. Preisinger & Leppert, M. (1988) Genetic alterations during colorectal-tumour development. *New England Journal of Medicine.* **319**:525-532.

Vogelstein, B., E. Fearon, S. Kern, S. Hamilton, A. Preisinger & Jessup, J. (1989). Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science.* **244**:207-211.

Wadler, S., B. Lembersky, M. Atkins, J. Kirkwood & Petrelli, N. (1990). Phase II trial of fluorouracil and recombinant interferon alfa-2a in patients with advanced clorectal carcinoma: an Eastern Cooperative Oncology Group Study. *Journal of Clinical Oncology.* **9**:1806-1810.

Wadler, S., E. Schwartz, M. Goldman, A. Lyver, M. Rader, M. Zimmerman, L. Itri, V. Weinberg & Wiernik, P. (1989) Fluorouracil and recombinant alfa-2a-interferon: an active regimen against advanced colorectal carcinoma. *Journal of Clinical Oncology.* **7**:1769-1775.

Wadler, S. & Weirnik, P. (1990). Clinical update on the role of fluorouracil and recombinant interferon alfa-2a in the treatment of colorectal carcinoma. *Seminars in Oncology.* **17**:16-21.

Wadler, s., R. Wersto & Weinberg, V. (1990). Interaction of fluorouracil and interferon in human colon cancer cell lines: cytotoxic and cytokinetic effects. *Cancer Res.earch* **50**:5735-5740.

Washtein, W.(1981). Thymidylate synthetase levels as a factor in 5-fluorodeoxuridine and Methotrexate cytotoxicity in gastrointestinal tumour cells. *Molecular Pharmacology.* **21**:723-728.

Washtein, W. (1983). Increased levls of thymidylate synthase in cells exposed to 5-fluorouracil. *Molecular Pharmacology.* **25**:171-177.

Washtein, W. (1984). Comparison of 5-fluorouracil metabolism in two human gastrointestinal tumor cell lines. *Cancer Research.* **44**:909-914.

Washtein, W.(1984). Increased levels of thymidylate synthetase in cells exposed to 5-fluoruracil. *Molecular Pharmacology.* **25(1)**:171-177.

Weh, H., D. Platz, D. Braumann, P. Buggish, N. Eckardt, W. Schmeigel, S. Drescher, U. Kleeberg, U. Mullerleile & Crone-Munzebrock, W. (1992). Phase II trial of 5-fluorouracil and recombinant interferon and continuous infusion fluorouracil in previously untreated metastatic colorectal adenocarcinoma. *European Journal of Cancer.* **28A**:1820-1823.

Welch, D., J. Bisi, B. Miller, D. Conaway, E. seftor, K. Yohem, L. Gilmore, R. Seftor, M. Nakajimaand & Hendrix, M. (1991). Characterisation of a highly invasive and spontaneously metastatic human malignant melanoma cell line. *International Journal of Cancer.* **47**:227-237.

Whitehead, T. (1979). Analytical luminescence: its potential in the clinical laboratory. *Clinical Chemistry.* **25(9)**:1531-1546.

Wohlheuter, R., R. McIvorand P. Plagemann. (1980). Facilitated transport of Uracil and 5-Fluorouracil and permeation of orotic acid into cultured mammalian cells. *Journal of Cellular Physiology.* **104:**309-319.

Wolmark, N., H. Rockette, B. Fisher, D. Wickerman, C. Redmond, E. Fisher, J. Jones, E. Mamounasand Petrelli, N. (1993). The benefit of leucovorin-modulated fluorouracil as postoperative adjuvant therapy for primary colon cancer: results from National Surgical Adjuvant Breast and Bowel Project protocol. *Journal of Clincal Oncology.* **10**:1879-1887.

Wrighley, P., M. Slevin & Clark, P. (1984). Alpha-2 interferon (IFN) in combination with 5-fluorouracil (5-FU) for for advanced colorectal carcinomas. *Proceedings of the Society for Clinical Oncology.* **3**:146.

Wright, J., A. Dreyfuss, I. El-Magharbel, D. Trites, S. Jones, S. holden Rosowsky, A & Frei, I.E. (1989). Selective expansion of 5,10-Methylenetetrahydrofolate pools and modulation of 5-fluorouracil anti-tumour activity of Leucovorin *in vivo. Cancer Res.* **49**:2592-2596.

Yin, M. & Rustum, Y. (1991). Comparative DNA strand breakage induced by FUra and FdUrd in human ileocecal adenocarcinoma (HCT-8) cells: relevance to cell growth inhibition. *Cancer Communications*. **3**:45-51.

Yin, M., S. Zakrewski & Hakal, M. (1982). Relationship of cellular co-factor pools to the activity of 5-fluorouracil. *Molecular Pharmacology***23**:190-197.

York, M., F. Greco & Figlin, R. (1993). A randomised phase III trial comparing 5-FU with or without interferon-alfa-2a for advanced colorectal cancer. *Proceedings of the American Society of Clinical Oncology*. 12:200.

Yoshimura, A., Y. Kuwazuru, T. Furukawa, H. Yoshida, H. Yamada & Akiyama, S. (1990). Purification and tissue distribution of human thymidine phosphorylase; high expression in lymhocytes, reticulocytes and tumours. *Biochim. Biophys. Acta.* **1034**:107-113.

Zimmerman, M. & Seidenberg, J. (1964). Thymidine Phosphorylase and Nucleotide Deoxyribosyltransferase in Normal and Malignant tissues. *The Journal of Biological Chemistry*. **239**:2618-2612.