Increased sensitivity to the prodrug 5'-deoxy-5-fluorouridine and modulation of 5-fluoro-2'-deoxyuridine sensitivity in MCF-7 cells transfected with thymidine phosphorylase

AV Patterson1,2, H Zhang1, A Moghaddam1, R Bicknell1, DC Talbot1, IJ Stratford2 and AL Harris1

1ICRF Clinical Oncology Unit, Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, UK; 2MRC Radiobiology Unit, Chilton, Didcot, Oxon OX11 0RD, UK.

Summary Platelet-derived endothelial cell growth factor (PD-ECGF) is identical to human thymidine phosphorylase (dTdpase). The human MCF-7 breast cancer cell line was transfected with the dTdpase cDNA and expressed a 45 kDa protein that was detected with anti-dTdpase antibody. Cell lysates possessed elevated dTdpase activity and cells had up to 165-fold increased sensitivity to the prodrug 5'-deoxy-5-fluorouridine (5'-DFUR) in vitro. Sensitivity to 5-fluorouracil (5-FU) and 5-fluoro-2'-deoxyuridine (5-FdUrD) was unchanged. Recombinant dTdpase was shown to catalyse directly the phosphorolytic cleavage of 5'-DFUR to 5-FU. Exogenous thymidine (dTd) reversed the toxicity of 5-FdUrD on the parental line (1 μM dTd increased the IC50 value 1000-fold), but the dTd rescue was substantially modulated in the dTdpase-expressing clone 4 (1 μM dTd raised the IC50 value 3-fold). We observed a substantial 'bystander' killing effect when small proportions of dTdpase-expressing cells were mixed with parental MCF-7 cells. dTdpase activity was on average 27-fold higher in breast tumours than in normal breast. The levels in wild-type MCF-7 are similar to the low end of the tumour expression. Thus, in some tumours resistance to 5'-DFUR therapy could be due to low dTdpase activity, and transfection to raise the dTdpase levels within the broad tumour range or above it should markedly enhance the sensitivity to the prodrug. These results confirm that dTdpase is a major pathway in the metabolic activation of 5'-DFUR, and the bystander effect suggests that this may be a suitable enzyme for gene therapy-directed enzyme/prodrug activation therapy.

Keywords: thymidine phosphorylase; 5'-deoxy-5-fluorouridine; 5-fluoro-2'-deoxyuridine; drug sensitivity; MCF-7 cell line

Platelet-derived endothelial cell growth factor (PD-ECGF) was originally isolated from platelets through its unique mitogenic activity on endothelial cells (Miyazono et al., 1987; 1989). It has since been shown to be homologous to human thymidine phosphorylase and a product of the same gene (Furukawa et al., 1992). Thymidine phosphorylase (dTdpase) (EC 2.4.2.4) catalyses the reversible phosphorolytic cleavage of thymidine (dTd), deoxyuridine and their analogues to their bases and deoxyribosyl 1-phosphate (Itzsch et al., 1985; el Kouni et al., 1993). However, while evidence strongly implicates dTdpase in the metabolic activation of 5'-deoxy-5-fluorouridine (5'-DFUR) (Fujimoto et al., 1985), no study has definitively demonstrated that pure human dTdpase can phosphorolytically cleave the glycosidic bond of the prodrug 5'-DFUR to yield 5-fluorouracil (5-FU). We show here that elevated expression of dTdpase sensitises MCF-7 breast cancer cells to 5'-DFUR and this sensitisation is related to the capacity of dTdpase to cleave 5'-DFUR to 5-FU.

Breast, ovarian, colorectal and gastric cancers have been shown to express elevated levels of dTdpase relative to the normal surrounding tissue (Zimmerman et al., 1964; Yoshimura et al., 1990). Increased dTdpase activity has also been found in the plasma of cancer patients compared with healthy controls (Pauly et al., 1977, 1978). This tumour-associated elevation of dTdpase activity has been exploited clinically through use of the prodrug 5'-DFUR. Yet success has been limited, possibly because of the heterogeneity of dTdpase activity within this group of carcinomas. Greater exploitation of this 'enzyme-prodrug activation' model could be achieved through the application of gene therapy techniques to direct the tissue-specific expression of dTdpase (Vile et al., 1993a). For example, in vivo transfection of cDNA sequences by direct intratumoral injection induces a small proportion of the tumour cell population to transiently express the construct (Vile et al., 1993b).

We investigated the possibility that tumours expressing a low basal level of dTdpase activity might be further sensitised to 5'-DFUR through the transfection and expression of dTdpase, and whether elevated expression within a small proportion of the cell population could sensitise neighbouring tumour cells to the prodrug. Comparative sensitivities of the parental and transfected cell lines to 5'-DFUR, 5-FU and 5-fluoro-2'-deoxyuridine (5-FdUrD) were examined in the presence and absence of exogenous dTd. The presence of salvageable dTd within the microenvironment of a tumour could reduce the efficiency of the prodrug–enzyme system, since dTd will compete with its analogue 5'-DFUR for the active site of dTdpase, and limits its cytotoxic effects. Furthermore, dTdpase may enhance the toxicity of the active drug 5-FU, by deoxyribosyl transfer of 2'-deoxyribosyl 1-phosphate (Zimmerman et al., 1964; Krenitsky, 1968), producing the deoxynucleoside 5-FdUrD, which can form 5-FdUMP through the action of thymidine kinase, 5-FdUMP can inhibit thymidylylate synthase, restricting de novo synthesis of dTMP, and can ultimately be fraudulently incorporated into DNA (Schwartz et al., 1992). If the cytotoxic effects of 5-FU are mediated in part by this pathway, bioavailable dTd would diminish any inhibitory effects of thymidylylate synthase inhibition.

Materials and methods

Chemicals

Thymine, dTd, 5-FU, 5-FdUrD and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyletherazolium bromide) were purchased from Sigma (Dorset, UK). 5'-Deoxy-5-fluorouridine (5'-DFUR) was a kind gift from Dr Hideo Ishitsuka, Nippon Roche KK (Kanagawa, Japan).
Preparation of anti-dThdPase antibody

Recombinant dThdPase protein, expressed as a glutathione S-transferase (GST) fusion protein in *Escherichia coli*, was purified and proteolytically cleaved with thrombin to remove the GST leader peptide (Moghaddam et al., 1992). The cleaved protein was used to generate anti-dThdPase antisera. Adjuvant preparations, immunisations and bleeding of the animals were carried out using a standard rabbit immunisation protocol.

Cell lines

Human MCF-7 breast cell lines and clones 4 and 7 were grown in E4 modified minimal essential medium (prepared at ICRF, Clare Hall, Cambridge, UK) supplemented with 10% fetal calf serum and 4 mM glutamine. Cells were routinely screened and found free of *Mycoplasma*.

Transfection of dThdPase cDNA into MCF-7 cells

The details of generation and characterisation are to be published (H Zhang and R Bicknell, manuscript in preparation). Briefly, pSin plasmid vector containing full-length dThdPase DNA was introduced into MCF-7 cells by electroporation. Stable transfectants were selected by long-term incubation in G418.

Determination of doubling times of MCF-7 and cloned cell lines

Cells were incubated in 96-well format, at a density of 5 × 10⁴ cells per well. At the times indicated, cells were incubated with 0.1 mg (50 μl of 2 mg ml⁻¹) of MTT for 4 h, and cell number was determined by reference to a standard absorption curve of predetermined cell numbers for each cell line.

Quantitation of drug sensitivity

The modified MTT assay (Carmichael et al., 1987) was used to determine the dose-response curves of the parental and clone cell lines, using a multiwell spectrophotometer (Titrettek Multitaskan Plus MKII, Flow Laboratories). IC₅₀ values were determined relative to control wells containing no drug, using Deltasoft software (Biometallics, Princeton, NJ, USA). Cells were seeded at 5 × 10⁴ per well and left for 3 h before drug application. All incubations were 7 days.

Preparation of cell lysates

MCF-7 and cloned lines were harvested in exponential growth phase by trypsinisation, washed in phosphate-buffered saline (PBS), and sonicated in 50 mM Tris–HCl, 0.15 M sodium chloride buffer, pH 7.4, at 4°C. Suspension was centrifuged at 10 000 g for 15 min (4°C). Supernatants were stored in liquid nitrogen and assayed for dThdPase activity.

Preparation of breast tissue cytosols

Breast tissue was removed during primary biopsy and stored in liquid nitrogen until preparation. Samples were ground by pestle and mortar in the presence of liquid nitrogen before automated homogenisation in 50 mM Tris–HCl, 0.15 M sodium chloride buffer, pH 7.4, at 4°C. Cell debris was removed by spinning at 300 g for 10 min (4°C). The resulting supernatant was spun at 100 000 g for 40 min (4°C) and stored at −80°C.

Assay of dThdPase activity

Lysates were incubated for 16 h at 37°C in 10 mM dThd or 5′-DFUR and 10 mM potassium phosphate, pH 7.4. The reaction was terminated by addition of 0.7 ml of ice-cold sodium hydrosxide (500 mM for dThd substrate, 20 mM for 5′-DFUR substrate) to 0.3 ml of reaction mixture, to produce a final solution pH of 13.3 and 12 respectively. Quenched samples were kept on ice, and the conversion of dThd to thymine and 5′-DFUR to 5-FU were measured spectrophotometrically at 300 nm and 305 nm respectively (Schwartz, 1978; Choong and Lee, 1985). Optical densities were related to standard plots for known thymine and 5-FU concentrations. Protein content of the cell lysates and breast tumour and normal tissue cytosols were determined using the Bio-Rad protein dye assay and quantitated against high-grade BSA protein standard. dThdPase activity is expressed as nmol substrate converted per mg total cytosolic protein per hour.

Immunoblot analysis

Samples of cells harvested for enzyme assays were washed in PBS buffer containing 1 mM phenylmethylsulphonyl fluoride, 1 mM benzamide, 50 μg ml⁻¹ leupeptin and 50 μg ml⁻¹ soya-bean trypsin inhibitor. Cells were lysed in 1 ml of 2% SDS plus inhibitors in PBS at 65°C for 5 min. DNA was broken up with a fine-gauge needle passed up and down. Samples were stored at −20°C. Samples were resolved by 10% polyacrylamide gel electrophoresis, and proteins on the gel were electrophoretically transferred overnight to a nitrocelulose hybridisation transfer membrane. The membrane was washed with blocking buffer and incubated for 30 min with specific dThdPase rabbit antibody (dilution 1:500). After washing, horseradish peroxidase-conjugated goat anti-rabbit antibody was incubated, and the membrane was developed using the enhanced chemiluminescence Western blotting detection kit (Amersham, Buckinghamshire, UK).

Results

dThdPase expression in MCF-7 cells

Two clones, 4 and 7, were selected following transfection of MCF-7 cells with full-length dThdPase cDNA. Cell lysates were prepared to examine the relative of dThdPase activity of the parental and transfected cell lines. The release of thymine from dThd and 5-FU from 5-DFUR were monitored spectrophotometrically at 300 nm and 305 nm respectively (Schwartz; 1978; Choong and Lee, 1985). The observed enzyme activities of the lysates were compared with the in vitro sensitivity assays. The parental MCF-7 cells had some endogenous dThdPase activity, while clone 4 and clone 7 displayed a 90- and 7-fold increase in activity respectively (Table I). Subsequent Western immunoblot analysis of the cell lysates confirmed that the clones expressed elevated levels of a 45 kDa protein that was detected by an anti-dThdPase antibody (Figure 1). Although enzyme activity could be detected, Western blotting was not as sensitive and could not demonstrate dThdPase in the parental MCF-7 cells. Comparative immunohistochemical staining of the parental and clone cell lines with primary anti-dThdPase antibody labelled with swine anti-rabbit FITC-conjugated antibody revealed the localisation of the 45 kDa protein to be predominantly cytoplasmic in the clonal lines.

| Table 1 | dThdPase activities of parental and clonal cell line lysates, with respect to both dThd and 5′-DFUR phosphorolytic cleavage |
|---------|---------------------------------------------------------------|
| **Thymidine phosphorylase activity of cell lysates ± s.e.m.** | **mg⁻¹ protein h⁻¹** | **nmol 5′-FU released mg⁻¹ protein h⁻¹** |
| **Cell lines** | **38.2 ± 5.9** | **47 ± 11.2** |
| **Clone 4** | **3383 ± 133** | **3160 ± 187** |
| **Clone 7** | **269 ± 12.2** | **264 ± 19.4** |

dThdPase activity (nmol of thymine or 5-FU released per hour per mg of protein) was monitored spectrophotometrically. Each value represents the mean ± s.e.m. of at least three independent determinations. Clones 4 and 7 are sublines of MCF-7 cells transfected with dThdPase cDNA.
Figure 1 Western immunoblot of recombinant dThdPase, MCF-7 parental line, clone 4 and clone 7 with anti-dThdPase antibody. Both clones 4 and 7 are sublines of MCF-7 cells transfected with dThdPase cDNA in the pSV2 vector. Both clones were selected by long-term incubation in Geneticin. Lysates prepared from each were separated by electrophoresis on a 10% sodium dodecyl sulphate-polyacrylamide gel and transblotted onto a nitrocellulose hybridisation transfer membrane. The membrane was incubated sequentially in anti-dThdPase antibody and horseradish peroxidase-conjugated goat anti-rabbit antibody, and then developed using an enhanced chemiluminescence Western blotting detection kit.

Growth rates of cell lines

The mean doubling times of parental MCF-7, clone 4 and clone 7 cells were 51.4 ± 9.2, 77.1 ± 14.2 and 67.5 ± 12.7 h respectively. There was no significant difference in cellular growth rates, indicating that elevated dThdPase expression does not appear to affect the growth rate of the cells.

Drug sensitivity of parental and transfected cell lines

The drug sensitivity of the cells was determined using the MTT assay (Table II). The IC₅₀ values for 5-FU were not significantly different between the parental line and clones 4 and 7, being 1.03 ± 0.73 and 1.44 ± 0.44 μM respectively (Figure 2a). However, the IC₅₀ values of the prodrug 5′-DFUR, which is converted to 5-FU by dThdPase, were markedly different, being 17.3 ± 3.1 and 7.1 μM for the parental clone, clone 4 and clone 7 respectively (Figure 2b). The IC₅₀ ratios of clone 4 and clone 7 were 165 and 2.4 times higher than that of the parental line. The differing sensitivities of the cell lines were reflected in their relative levels of dThdPase activity with respect to the release of 5-FU from 5′-DFUR (Table I). Sensitivity to 5-FUDR was not significantly different between the parental and clonal lines.

Modulation of drug sensitivity by exogenous thymidine

The presence of salvageable dThd may circumvent any toxicity associated with the inhibition of de novo dTMP synthesis. Therefore we examined the capacity of physiologically relevant concentrations of dThd to modulate the toxicity of 5′-DFUR, 5-FU and 5-FUDR in vitro.

Co-addition of dThd during 5-FU exposure did not affect the sensitivity of either the parental or transfected cell lines, even at the maximum concentration (150 μM) which was non-toxic to the cells. This suggests that thymidylate synthase inhibition is not an important determinant for 5-FU toxicity in these cell lines (Danenberg et al., 1974; Kufe and Major, 1981). In contrast, physiologically relevant concentrations of dThd (1–10 μM) could partially reverse the inhibitory activity of 5′-DFUR on clones 4 and 7. Indeed, 10 μM dThd shifted the IC₅₀ value of clone 4 for 5′-DFUR by 18-fold (Figure 3a). Nevertheless, clone 4 cells were still markedly sensitised to 5′-DFUR compared with controls, and sufficiently high prodrug concentrations (≥ 10 μM) could overcome the dThd-induced reversal of toxicity. However, 1–10 μM dThd had no effect on the response of the parental line to 5′-DFUR (Figure 3b).

There was a marked capacity of exogenously added dThd (1–3 μM) to modulate the inhibitory effects of 5-FUDR in the parental cells which was significantly reversed in the clone 4 cells, particularly clone 4. This suggests that the non-phosphorylolytic activity of dThdPase can reduce the intracellular availability of dThd, reducing competition with 5-FdUTP for incorporation into DNA. Indeed, 1 μM dThd increased the IC₅₀ value of 5-FUDR for the parental line from 2.3 to approximately 2400 nM, some 1000-fold (Figure 4a), while producing only a 3-fold reversal of toxicity in clone 4, from 2.6 to 8 nM (Figure 4b).

Sensitisation of neighbouring cells

Addition of a small fraction of clone 4 cells, markedly sensitised neighbouring parental cells to the action of 5′-DFUR
MCF-7 cells transfected with dThdPase are sensitised to 5'-deoxy-5-fluorouridine

AV Patterson et al

672

Figure 3 (a) Representative in vitro dose–response curve of the dThdPase transfected clone 4 to the prodrug of 5-FU, 5'-deoxy-5-fluorouridine, in the absence (O) or presence of exogenously added thymidine, at a concentration of 1 μM (Δ), 3 μM (△) and 10 μM (○). Per cent growth inhibition is relative to drug-free controls and determined using the MTT assay. Error bars represent the s.d. of eight wells. (b) Representative in vitro dose–response curve of the parental MCF-7 cell line to the prodrug of 5-FU, 5'-deoxy-5-fluorouridine, in the absence (O) or presence of exogenously added thymidine, at a concentration of 1 μM (Δ), 3 μM (△) and 10 μM (○). Per cent growth inhibition is relative to drug-free controls and is determined using the MTT assay. Error bars represent the s.d. of eight wells.

Figure 4 (a) Representative in vitro dose–response curve of the parental MCF-7 cell line to 5-fluoro-2'-deoxyuridine, in the absence (O) or presence of exogenously added thymidine, at a concentration of 1 μM (Δ) and 3 μM (△). Per cent growth inhibition is relative to drug-free controls and is determined using the MTT assay. Error bars represent the s.d. of eight wells. (b) Representative in vitro dose–response curve of the dThdPase-transfected clone 4 cell line to 5-fluoro-2'-deoxyuridine, in the absence (O) or presence of exogenously added thymidine, at a concentration of 1 μM (Δ) and 3 μM (△). Per cent growth inhibition is relative to drug-free controls and is determined using the MTT assay. Error bars represent the s.d. of eight wells.

(Figure 5). The IC₅₀ of a population containing a 20:80 mixture of clone 4 and parental cells was reduced 10-fold. This represents a significant in vitro ‘bystander’ killing effect at a concentration at which the parental line is refractory to the effects of 5'-DFUR.

dThdPase activity of normal and malignant breast tissue

Considerable heterogeneity was found in both the normal and tumour cytosol samples, although dThdPase activity was consistently elevated in the breast tumour cytosols (P<0.0002). Values ranged from 46.5 to 929 nmol h⁻¹ mg⁻¹ (median 273 nmol h⁻¹ mg⁻¹), while normal tissue cytosols displayed a more modest variability, ranging from 1.6 to 47 nmol h⁻¹ mg⁻¹ (median 10.6 nmol h⁻¹ mg⁻¹). However none of the breast tumour cytosols showed elevations in dThdPase activity of the order of that found for clone 4, whilst clone 7 represents the levels at the upper third of the tumour dThdPase range (Figure 6). dThdPase activity did not correlate with oestrogen receptor (ER) or epidermal growth factor receptor (EGFR) status in either the tumour or normal tissue samples.

Discussion

Expression of dThdPase is elevated in many malignant tumours, but a wide range of activities have been reported (Zimmerman et al., 1964; Yoshimura et al., 1990). This heterogeneity was confirmed by dThdPase enzyme assay of a sample group of breast tumour cytosols prepared from excision biopsies. We transfected dThdPase into a breast cancer cell line to reproduce the range found in human breast tumours and assess its contribution to drug resistance and potential gene therapies. dThdPase activity per mg of total cytosolic protein in the breast tumour samples showed a 20-fold range of elevated activities, which were consistently greater (mean 27-fold) than that found for normal breast tissue cytosols (Figure 6).

Two of the selected clones had elevated levels of dThdPase
activity and expressed a 45 kDa protein that was detected with anti-dThdPase antibody. Increased expression of this enzyme sensitised the human MCF-7 breast cell line to 5'-DFUR in vitro. Clone 4, which showed a 96-fold increase in dThdPase activity (with respect to the release of thymine from dThd), had a 165-fold reduced IC50 value for 5'-DFUR compared with the parental line. Conversion of the prodrug 5'-DFUR to 5-FU by the cell lysate preparation of clone 4 was 67-fold greater than that of the parental clone. Clone 7 had a 2.4-fold differential in the IC50 value for 5'-DFUR relative to the parental line. However, this difference was also reflected in the ability of the cell lysate to catalyse the formation of 5-FU from 5'-DFUR, being 5.6-fold greater than that of the parental line. The degree of sensitivity appears to be related to the capacity of the dThdPase to phosphorolytically cleave the prodrug 5'-DFUR to yield the metabolically active drug 5-FU. An exact correlation was not obtained, probably because of variables in the different assays (e.g. cell extracts dThdPase activity is assayed over 16 h in vitro sensitivity over 7 days). Comparison of our observations of the relative increases in dThdPase expression in tumour samples in relation to our in vitro results indicates that an exploitable therapeutic differential exists between normal and tumour tissue with respect to 5'-DFUR treatment, but the heterogeneity of overexpression in malignant tissue suggests that tumour dThdPase profiling could be an important component of patient selection programmes.

Circulating dThd is present in the plasma of individuals at 0.1–0.2 μM (Shaw et al., 1988a,b). While the degree and extent of vascularisation of a solid tumour largely dictates the bioavailability of such nutrients, dThd availability in the microenvironment of a tumour may become elevated as a result of release from dying cells. Such increased bioavailability of dThd could modulate the efficacy of the prodrug 5'-DFUR by inhibiting the dThdPase-mediated cleavage to the active agent, 5-FU. However, we showed that even levels of dThd 50- to 100-fold greater than those detectable in plasma could not fully reverse the effect of 5'-DFUR, and 1 μM dThd had only a marginal effect. The cytotoxic effects of 5-FU are thought to be mediated, in part, by the inhibition of thymidylate synthase, through the anabolism of 5-FU to 5-FdUMP (Danenberg et al., 1974; Santi and McHenry, 1972). However, the addition of 150 μM dThd did not influence the toxicity of 5-FU in the MCF-7 cell line, indicating that this is not an important mechanism of toxicity for MCF-7 cells (Kufe and Major, 1981). Thus the observed reversal of 5'-DFUR toxicity by dThd in the clone 4 and 7 cell lines is mediated at the level of competition for prodrug activation, rather than modulating the cytotoxicity of the released 5-FU.

The IC50 values of 5-FUdR for the parental and transfected cell lines were not significantly different, suggesting that 5-FUdR is probably not an important substrate for the phosphorolytic activity of dThdPase. However, the dThdPase activity of the clones could significantly reverse the capacity of dThd to rescue the cells from the toxic effects of 5-FUdR (Nayak, 1992). This suggests that the phosphorolytic breakdown of dThd by dThdPase renders it metabolically unavailable to bypass the inhibition of thymidylate synthase or to ultimately compete with 5-FdUTP for incorporation into DNA. Thus, it is possible that in vivo levels of dThdPase could contribute to 5-FUdR response. If so, these cases may respond well to 5'-DFUR treatment.

The requirement for dThdPase in the sensitisation to 5'-DFUR has recently been confirmed by transfecting dThdPase into human KB epidermoid carcinoma cells (Haraguchi et al., 1993). We furthered this observation by establishing that recombinant dThdPase can catalyse the phosphorolytic cleavage of 5'-DFUR to release 5-FU. This takes account of potential differences between substrate specificity for the thymine-2'-deoxyriboside and the 5-fluorouracil-5'-deoxyribose, and demonstrates a direct role for dThdPase in the sensitisation to 5'-DFUR. In contrast to the MCF-7 cell line, the KB epidermoid parental line expressed no endogenous dThdPase activity and the level of dThdPase activity conferred upon the clone by transfection was relatively low (168 nmol h-1 mg-1). This was reflected in the 19-fold differential in IC50 values for 5'-DFUR. The transfection of dThdPase into a cell line which has some endogenous dThdPase activity, to sensitize the carcinoma cells further, more accurately reflects the potential in vivo situation with respect to enhancing the sensitivity of a tumour mass in situ, through delivery of the dThdPase cDNA sequence under the control of a suitable tissue-specific promoter. Such an approach may help to overcome the heterogeneity of elevated dThdPase expression observed in some malignant tissues. A significant 'bystander' killing effect was observed for 5'-DFUR in the mixing experiments, suggesting that the
active drug, 5-FU, can diffuse from its site of formation and exert its effects upon neighbouring cells in vitro. It has been suggested that the main pathway for the bystander effect is via gap junctions (Freeman et al., 1993), and this is the case for phosphorylated metabolites (Bi et al., 1993). However 5-FU can diffuse via a facilitated transporter, which may be an advantage if gap junctions are down-regulated. Therefore, the targeting of a tumour mass with a tissue-specific promoter-driven dThdPase sequence in vivo may not require the transduction of every tumour cell for effective killing of neighbouring cells to occur. Advances in the efficiency of gene delivery through the use of techniques such as receptor-mediated endocytosis and replication-incompetent adenovirus co-internalisation (Cotten et al., 1992; Christiano et al., 1993) may make dThdPase a suitable gene for prodrug therapies. Potentially more important, such delivery protocols have resulted in very favourable increases in the level of expression of reporter genes. Comparative analysis of the parental and transfected lines' response to 5'-DFUR, and their differing levels of dThdPase expression, suggests that increased levels of expression may result in a considerable therapeutic gain in vivo.

Another prodrug–enzyme-activated model, using the expression of cytosine deaminase to release 5-FU from the prodrug 5-fluorocytosine (Huber et al., 1993), illustrates the marked therapeutic advantages that can be achieved with such approaches in vivo. However the 5'-DFUR dThdPase model may prove to be superior since co-metabolism of endogenous dThd, although in direct substrate competition with 5'-DFUR, if present at 10- to 100-fold physiological excess (1–10 μM), could nevertheless enhance the cytotoxicity of the activated drug in a number of ways. Phosphorylative cleavage of dThd by dThdPase would render it metabolically unavailable to bypass the inhibition of de novo synthesis and to compete with FdUTP for incorporation into DNA (Major et al., 1982). The ‘thymidine-less’ state resulting from the inhibition of thymidylate synthetase by FdUMP would make tissues expressing dThdPase sensitive to the depletion of salvageable dThd, limiting any potential ‘escape’ from the dThd-less-induced stress and its associated cytotoxicity (Houghton et al., 1993). Furthermore, dThdPase may enhance the formation ofFdUMP through the reversible addition of deoxyribosyl 1-phosphate to the enzymatically released 5-FU (Schwartz et al., 1994). Depletion of the available dThd would also serve to increase local concentrations of thymine, which would competitively inhibit the catabolism of 5-FU by dihydrouracil dehydrogenase, potentially extending its half-life within the tumour mass (Santelli and Valeroti, 1980). Prolonging the duration and intensity of tumour tissue exposure to 5-FU, and its associated anabolics, has been shown to limit the occurrence of resistant clones associated with suboptimal chronic exposures in vitro (Sobrero et al., 1993). This may have implications in restricting the development of acquired resistance in vivo. Increasing the duration of 5-FU exposure has also been shown to enhance significantly the cytotoxicity of the biomodulators leucovorin and interfering α2a in vitro (Houghton et al., 1993).

5'-DFUR, but not 5-FU, also possesses other antiproliferative-independent characteristics which may prove clinically advantageous. For example it has anti-cachectic activity (Tanaka et al., 1990; Eda et al., 1991) and has been reported to inhibit metastases in an artificial murine Lewis lung carcinoma metastasis model (Berman, 1995).

In conclusion, our results show that dThdPase is a candidate gene for gene-directed enzyme prodrug therapy, and cell lines with endogenous dThdPase can be further sensitised to 5'-DFUR. This approach could also overcome one mechanism of 5-FUDR resistance.

These data also suggest that selection of patients for 5'-DFUR therapy based on tumour levels of dThdPase should be considered.

References

BERTRAM JS. (1995). Fifth Heidelberg conference on targets for cancer research: prevention, differentiation, and selective therapy. Cancer Res., 55, 705–709.

B1 W. PARYSELM, S. WARMNICK R AND STAMBOOPI PJ. (1993). In vitro evidence that metabolic cooperation is responsible for the bystander effect in tumours observed with HSV tk retrovial gene therapy. Human Gene Ther., 4, 725–731.

CARMICHAEL J. DEGAKKAG W. GAZDAR AF. MINNA JD & MITCHELL JB. (1987). Evaluation of a teratoma-based semiautomated colormetric assay: assessment of chemosensitivity testing. Cancer Res., 47, 936–942.

CHOONG YS AND LEE SP. (1985). The degradation of 5'-deoxy-5-fluorouridine by pyrimidine nucleoside phosphorylase in normal and cancer tissue. Clin. Chim. Acta, 149, 175–183.

COTTEN M. WAGNER E. ZATLOUKAL K. PHILLIPS S. CUIREL DT AND HALL DE. (1992). High-level gene delivery of small and large (48 kilobase) gene constructs using the endosom-disruption activity of defective or chemically inactivated adenovirus particles. Proc. Natl Acad. Sci. USA, 89, 6094–6098.

CRISTIANO RJ. SMITH LC AND WOO SL. (1993). Hepatic gene therapy: adenovirus enhancement of receptor-mediated gene delivery and expression in primary hepatocytes. Proc. Natl Acad. Sci. USA, 90, 2122–2126.

DANENBERGER F. LANGENBACH RJ AND HEIDELBERGER, C. (1974). Structures of reversible and irreversibly complexed thymidylate synthetase and fluorinated pyrimidine nucleotides. Biochemistry, 13: 926–930.

EDA H. TANAKA Y AND ISHTSUKA H. (1991). 5'-Deoxy-5-fluorouridine improves cachexia by a mechanism independent of its antiproliferative action in colon 26 adenocarcinoma-bearing mice. Cancer Chemother. Pharmacol., 29, 1–6.

FREEMAN SM. ABBOLD CN. WHARTENBY KA. PACKMAN CH. KOEPLIN DS. MOOLTEN FL AND ABRAHAM G. (1993). The ‘bystander effect’: tumour regression when a fraction of the tumour mass is genetically modified. Cancer Res., 53, 5274–5283.

FUJIMOTO S. WANG Y. INOUE K. AND OGAWA M. (1985). Antitumour activity of a new fluoropyrimidine derivative, 5'-deoxy-5-fluorouridine, against murine and human experimental tumours. Jpn J. Cancer Res., 76, 644–650.

FURUKAWA T. YOSHIHARA A. SUMIZAWA H. HAGURUCI M. AND AKIYAMA SI. (1992). Antiangiogenic factor. Nature, 356, 668.

HARAGUCHI M. FURUKAWA T. SUMIZAWA H. AND AKIYAMA S. (1993). Hypoxia-sensitised gene expression and large cell complexing platelet-derived endothelial cell growth factor to pyrimidine anti metabolites. Cancer Res., 53, 5680–5682.

HOUTHAGEN JA. MORTON CL. ADKINS DA AND RAHMAN A. (1993). Coexpression of the interaction among 5-fluorouracil, leucovorin, and interferon-α2a in colon carcinoma cells. Cancer Res., 53, 4243–4250.

HUBER BE. AUSTIN EA. GOOD SS. KNICH VC. TIBBELS S AND RICHARDS CA. (1993). In vivo antitumour activity of 5-fluorouracil on human colorectal carcinoma cells genetically modified to express cytosine deaminase. Cancer Res., 53, 4619–4626.

ILTZSCH MH. KOUNI MH AND CHA S. (1984). Antitumour activity of thymidine phosphorylase from mouse liver. Biochemistry, 24, 7999–8007.

EL KOUNI MH. EL KOUNI MM AND NAGUIB F. (1993). Differences in activities and substrate specificity of human and murine pyrimidine nucleoside phosphorylases: Implications for chemotherapy with 5-fluoropyrimidines. Cancer Res., 53, 3687–3693.

Abbreviations: PD-ECGF, platelet-derived endothelial cell growth factor; dThdPase, thymidine phosphorylase; 5-FU, 5-fluorouracil; 5'-DFUR, 5'-deoxy-5-fluorouridine, (doxifluridine, Furtulan); 5-FUdR, 5-fluoro-2'-deoxyuridine; 5'-DFURdUMP, 5-fluoro-2'-deoxyuridine 5'-monophosphate; 5'-DFUdUTP, 5-fluoro-2'-deoxyuridine 5'-triphosphate; dThd, thymidine; dTMP, thymidine 5'-monophosphate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; IC50, concentration of drug at which cell growth is inhibited by 50%; SDS, sodium dodecyl sulphate; cDNA, complementary DNA.
KRENTSKY TA. (1968). Pentosyl transfer mechanisms of the mammalian nucleoside phosphorylase. *J. Biol. Chem.*, 243, 2871–2875.

KUFE DW and MAJOR PP. (1981). 5-Fluorouracil incorporated into human breast carcinoma RNA correlates with cytotoxicity. *J. Biol. Chem.*, 256, 9802–9805.

MAJOR PP, EGAN E, HERRICK D AND KUFE DW. (1982). 5-Fluorouracil incorporation in DNA of human breast carcinoma cells. *Cancer Res.*, 42, 3005–3009.

MIYAZONO K, OKABE T, URABE A, TAKAKU F AND HELDIN CH. (1987). Purification and properties of an endothelial cell growth factor from human platelets. *B. Biol. Chem.*, 262, 4098–4103.

MIYAZONO K AND HELDIN CH. (1989). High-yield purification of platelet-derived endothelial cell growth factor: Structural characterisation and establishment of a specific antiserum. *Biochemistry*, 28, 1704–1710.

MOGHADDAM A AND BICKNELL R. (1992). Expression of platelet-derived endothelial cell growth factor in *Escherichia coli* and confirmation of its thymidine phosphorylase activity. *Biochemistry*, 38, 12141–12146.

NAYAK R. (1992). Thymidine inhibits the incorporation of 5-fluoro-2'-deoxyuridine into DNA of mouse mammary tumours. *Biochem. Biophys. Res. Commun.*, 184, 467–470.

PAULY JL, SCHULLER MG, ZELCER AA, KIRSS TA, GORE SS AND GERMAIN MJ. (1977). Identification and comparative analysis of thymidine phosphorylase in the plasma of healthy subjects and cancer patients: brief communication. *J. Natl Cancer Inst.*, 58, 1587–1590.

PAULY JL, PAOLINI NS, EARB RL AND GERMAIN MJ. (1978). Elevated thymidine phosphorylase activity in the plasma and ascitic fluids of tumor-bearing animals. *Proc. Soc. Exp. Biol. Med.*, 157, 262–267.

SANTCELL G AND VALERIOTHE F. (1980). In vivo potentiation of 5-fluorouracil cytotoxicity against AKR leukemia by purines, pyrimidines, and their nucleosides and deoxynucleotides. *J Natl Cancer Inst.*, 64, 69–72.

SANTCLDV AND MCHENRY CS. (1972). 5-Fluoro-2'-deoxyuridylate: covalent complex with thymidylate synthase. *Proc. Natl Acad. Sci. USA*, 69, 1855–1857.

SCHWARTZ EL, HOFFMANN M, O'CONNOR CJ AND WADLER S. (1992). Stimulation of 5-fluorouracil metabolic activation by interferon in human colon carcinoma cells. *Biochem. Biophys. Res. Commun.*, 182, 1232–1239.

SCHWARTZ EL, BAPTISTE N, O'CONNOR CJ, WADLER S AND OTTER BA. (1994). Potentiation of 5-fluorouracil in colon carcinoma cells by the combination of interferon and deoxyribonucleotides results from complementary effects on thymidine phosphorylation. *Cancer Res.*, 54, 1472–1478.

SCHWARTZ M. (1978). Thymidine phosphorylase from *Escherichia coli*. *Methods Enzymol.*, 51, 442–445.

SHAW T, SMILLIE RH AND MACPHEE, D.G. (1988a). The role of blood platelets in nucleoside metabolism: assay, cellular location and significance of thymidine phosphorylase in human blood. *Mut. Res.*, 200, 99–116.

SHAW T, SMILLIE RH AND MACPHEE, D.G. (1988b). The role of blood platelets in nucleoside metabolism: regulation of thymidine phosphorylase. *Mutat. Res.*, 200, 117–131.

SOBRERO AF, ASCHELE C, GUGLIELMI AP, MORIZ AM, MELIOLI GG, ROSSO R AND BERTINO JR. (1993). Synergism and lack of cross-resistance between short-term and continuous exposure to fluorouracil in human colon adenocarcinoma cells. *J. Natl Cancer Inst.*, 23, 1937–1944.

TANAKA Y, EDA H, FUJIMOTO K, TANAKA T, ISHIKAWA T AND ISHITSUKA H. (1990). Antiapoptotic activity of S'-deoxy-5-fluorouridine in a murine tumor cachexia model, colon 26 adenocarcinoma. *Cancer Res.*, 50, 4528–4532.

VILE RG AND HART IR. (1993a). In vitro and in vivo targeting of gene expression to melanoma cells. *Cancer Res.*, 53, 962–967.

VILE RG AND HART IR. (1993b). Use of tissue-specific expression of the herpes simplex virus thymidine kinase gene to inhibit growth of established murine melanomas following direct intratumoural injection of DNA. *Cancer Res.*, 53, 3860–3864.

YOSHIMURA A, KUWAZURU Y, FURUKAWA T, YOSHIDA H, YAMADA K AND AKIYAMA S. (1990). Purification and tissue distribution of human thymidine phosphorylase: high expression in lymphocytes, reticulocytes and tumours. *Biochem. Biophys. Acta.*, 1034, 107–113.

ZIMMERMAN M AND SEIDENBERG J. (1964). Deoxynucleosyl transfer. Thymidine phosphorylase and nucleoside deoxynucleosyltransferase in normal and malignant tissue. *J. Biol. Chem.*, 230, 2618–2621.