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ORIGINAL ARTICLE

An evaluation of thymidine phosphorylase as a means of preventing thymidine rescue from the thymidylate synthase inhibitor raltitrexed

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Abstract The antitumour effect of thymidylate synthase inhibitors such as raltitrexed (RTX) may be reversed by salvage of thymidine (Thd). Since thymidine phosphorylase (TP) depletes Thd, the potential for tumour-selective depletion of Thd using antibody-mediated delivery of TP to tumours was investigated. In vitro studies demonstrated that 25×10^{-3} units/ml TP depleted extracellular Thd (3 μM) and restored sensitivity to the growth inhibitory effects of RTX in Lovo and HT29 cell lines. Thymidine concentrations in xenograft tumours were inversely proportional to the activity of TP in the tumour, and the presence of a subcutaneous Lovo xenograft reduced plasma Thd concentrations from 0.92 ± 0.07 to 0.37 ± 0.04 μM. Intravenous administration of native TP enzyme depleted plasma Thd to 5 nM, but following rapid elimination of TP, plasma Thd returned to pretreatment values. There was no effect on tumour TP or Thd. Conjugation of TP to the A5B7 F(ab)2 antibody fragment, which targets carcinoembryonic antigen (CEA) expressed on colorectal cell-lines such as Lovo, did result in selective accumulation of TP in the tumour. However, there was no tumour-selective depletion of Thd and there did not appear to be any potential benefit of combining antibody-targeted TP with RTX.

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Introduction

Thymidylate synthase (TS) remains an important target for anticancer treatment, as reflected in the recent introduction of pemetrexed for mesothelioma and non-small cell lung cancer, and the continuing importance of 5-fluoropyrimidines in various tumour types. Raltitrexed (RTX), is a potent and selective quinazoline antifolate TS inhibitor [8] which, after entering the cell via the reduced folate carrier (RFC), undergoes rapid polyglutamation resulting in increased cellular retention and increased affinity for TS [6]. The inhibition of TS leads to a depletion of dTMP, resulting in misincorporation of dUTP into DNA during synthesis or repair, and ultimately leading to lethal DNA damage [25]. In vivo, RTX has shown antitumour activity in mice bearing L1210:ICR tumours [8] and in a clinical trial of 176 patients with advanced colorectal cancer, patients treated with RTX at a dose of 3 mg/m^2 as a 15-min infusion every 3 weeks had a 26% objective response rate, with a further 30% having minor responses [26].

The cytotoxic effects of RTX and other TS inhibitors may be reversed by salvageable thymidine (Thd), which enters the cell via nucleoside transporters [16] and is phosphorylated by thymdine kinase (TK) to form dTMP. In the presence of a TS inhibitor, extracellular Thd is consumed either by catabolism or by uptake into cells followed by incorporation into DNA. Since the pool of extracellular Thd is ultimately finite, and when TS is inhibited fully each cell doubling requires an exponential increase in Thd salvage [7],

depletion of extracellular Thd offers an approach to overcoming a potential mechanism of resistance to TS inhibitors such as RTX.

Thymidine phosphorylase (TP) catalyses the phosphorolysis of Thd, deoxyuridine and their analogues to the respective base and 2-deoxyribose-1-phosphate. This essentially irreversible reaction has the potential to deplete salvageable Thd, as the utilization of thymine by cells is generally less than 1% of Thd incorporation [5, 19]. Exogenous *E. coli* TP has been shown to restore the activity of RTX against MCF-7 cells in the presence of 3 μM Thd [14], demonstrating that exogenous TP is able to restore RTX-induced growth inhibition in the presence of salvageable Thd.

While depletion of Thd by TP is feasible in vitro, the systemic administration of TP may result in Thd depletion in normal tissue compartments and thereby lead to an increase in TS inhibitor toxicity. However, using antibodies to achieve tumour localization of TP and so selectively deplete Thd in the tumour is potentially attractive. Previous studies have used the A5B7 antibody, targeted to the tumour-selective carcinoembryonic antigen (CEA), in order to deliver enzymes to CEA-expressing tumours [1, 3, 20]. Furthermore, the $F(ab)_2$ fragment of the A5B7 antibody produced enhanced tumour to blood enzyme activity ratios in the LS174T tumour xenograft model, as compared with conjugates generated from the intact antibody [15].

The aim of the studies presented here was to evaluate the influence of endogenous and exogenous TP activity on Thd concentrations in vitro and to determine the effect of Thd depletion on sensitivity to RTX in human colorectal cancer cells. In addition, in vivo studies were performed using un-conjugated TP and TP conjugated to A5B7 $F(ab)_2$ to determine if TP can be delivered specifically to the tumour, as evaluated by measuring enzyme activity and tumour Thd levels. The LoVo xenograft tumour model was used, as it is documented to express high levels of CEA, the antigen recognized by A5B7 $F(ab)_2$, in addition to HT29 and TP-transfected HT29 cell lines.

Materials and methods

Chemicals and cell lines

RTX was a gift from AstraZeneca (Alderly Edge, Cheshire, UK). Thd and thymine were obtained from Sigma-Aldrich (Gillingham, Dorset, UK). TP and A5B7-$F(ab)_2$-TP were provided by Protherics (Porton Down, Wiltshire, UK). The inhibitor of TP (TPI; 5-chloro-6-(1-(2-iminopyrrolidinyl)methyl) uracil hydrochloride) was a gift from Philip Reigan, University of Manchester (Manchester, UK). Human HT29 and TP transfected HT29(TP) colorectal carcinoma cell lines, were obtained from Dr J Plumb, University of Glasgow (Glasgow, Scotland, UK) and were grown in DMEM medium (Gibco BRL, Paisley, Scotland, UK). The LoVo human colorectal carcinoma cell line was grown in RPMI 1640 medium (Gibco BRL) supplemented with 10% (v/v) foetal calf serum and 1,000 units/ml penicillin and 100 μg/ml streptomycin (Gibco BRL).

Growth inhibition assay

Growth inhibition was measured using the sulforhodamine B (SRB) assay [21]. Cells exposed to the different drug and/or enzyme concentrations for three cell doublings were fixed with 50% (w/v) TCA and washed with deionized water. Cells were stained with 0.4% (w/v) SRB dissolved in 1% (v/v) acetic acid (Fisher Scientific UK, Loughborough, Leics, UK) for 30 min, washed with acetic acid and the stain solubilized in 10 mM Tris base (BDH, Poole, Dorset, UK) pH 10.5 by shaking gently for 20 min. Plates were then read on a computer-interfaced Dynatech MR7000 plate reader (Dynatech, Billinghurst, West Sussex, UK) at 570 nm. All in vitro experiments were performed in medium containing fetal calf serum. The concentration of thymidine in the medium was negligible (≤ 0.2 μM) for these experiments.

Cells were seeded in 96 well plates (1×10^3/well) and were exposed to RTX for three cell doublings to determine the IC_{50} and IC_{90} concentrations. Cells treated at both IC_{50} and IC_{90} concentrations of RTX were grown in medium supplemented with increasing concentrations of Thd (0.1, 0.3, 1, 2 and 3 μM) to determine the concentration of Thd required for the reversal of RTX-induced growth inhibition. Medium was then supplemented with the concentration of Thd thereby determined (3 μM) and RTX (IC_{50} and IC_{90} concentrations) in the presence of increasing concentrations of TP, in order to determine the concentration of TP required for restoration of RTX sensitivity. TP activities and Thd concentrations in cell lysates were determined as described below.

TP activity assay

For the analysis of TP activity in cell lines, a cell pellet of 2×10^8 cells were placed in 1 ml of lysis buffer (1% w/v aprotinin and 1% w/v tergetol in PBS) and sonicated using the Soniprep 150 (Sanyo Biomedical

Europe, Loughborough, Leics, UK) for two 10 s bursts, following which the solution was microfuged at 10,000*g* for 3 min. The supernatant was collected for analysis by HPLC.

Samples were analysed using reverse phase HPLC on a Nucleosil 100 C_{18} 3 μm 4.6 × 100 mm column (Jones Chromatography). One hundred microlitres of lysate were placed in sample vials containing 100 μM Thd in 5 mM sodium phosphate pH 7. Sample vials were placed in an autosampler (Waters Alliance integrated solvent delivery system, Waters, Watford, Hertfordshire, UK) at 37°C. One hundred microlitres were injected and eluted from the HPLC column using an isocratic mobile phase of 0.05 M ammonium acetate pH 4.5 containing 3% v/v acetonitrile at a flow rate of 1.5 ml/min over 4 min. Repeated injections from each sample were analysed every 5 min over a 25-min period. Thymine and thymidine were detected using a Waters 996 photodiode array detector at a wavelength of 260 nm. Thymine standards prepared in 5 mM sodium phosphate pH 7 at 37°C were analysed to create a standard curve (0.1–100 μM thymine). TP activity was determined as rate of thymine formation in nmol/min/mg of protein as determined by the BCA protein assay (Sigma, Poole, UK), and was expressed as units/mg protein (1 unit forms 1 μmol thymine/min). The assay was linear over the range of standards, which were reproducible within 10% on repeat analysis within and between assays.

In vivo determination of Thd in plasma and tumour

For xenograft studies, 1×10^7 LoVo cells were implanted into female athymic nude mice (CD1 nu/nu, Charles River UK, Ramsgate, Kent, UK). Treatment groups included 5 or 10 mice at each time point. Mice were treated with TP or A5B7-F(ab)$_2$-TP as detailed below, and blood and tumour samples removed 0, 2, 6, 24 and 48 h after injection. Plasma was separated immediately and 100 μl were placed into disposable borosilicate tubes on ice. Five hundred microlitres of acetonitrile were added to the tube whilst vortex mixing and then the tube was vortexed for a further 20 s. The supernatant was removed and placed in a fresh borosilicate tube and evaporated to dryness under nitrogen in a Turbovap LV solvent evaporator (Zymark Instruments, Runcorn, Cheshire, UK) at 37°C. The residue was resuspended in 300 μl of 1% (v/v) acetic acid (Fisher) and vortex mixed for 20 s. One hundred and fifty microlitres were transferred to an LCMS small volume insert and placed in an autosampler (Perkin Elmer Limited, Pangbourne, Berkshire, UK) at 4°C. Tumours were placed in 500 μl of PBS and left on ice for 30 min, then homogenized (Ultra Turrax T25, Janke and Kunhal GMBH, Germany). The homogenate was microfuged at 13,000*g* for 5 min at room temperature and analysed for Thd as described above for plasma.

Samples were analysed on an Applied Biosystems API2000 (Applied Biosystems, Warrington, UK) liquid chromatography mass spectrometer. One hundred microlitres of sample were analysed using a Nucleosil ODS 3 μm 100 × 4.6 mm column (Jones Chromatography, Pontypridd, Mid-Glamorgan, Wales, UK) and a Genesis C_{18} 20 × 4 mm 3 μm cartridge pre-column. Two pumps were used to generate a gradient from 100% acetic acid 1% (v/v) for 6 min, to 50% methanol over 2 min. This was maintained for 2 min, returning to 100% aqueous over 1 min, with re-equilibration for a further 7 min, to give a total run time of 18 min at a flow rate of 0.5 ml/min. The mass-spectrometer was set to multiple reaction monitoring (MRM) in ES+ mode, with parent and fragment ions of *m*/*z* 247 and 127, respectively. The calibration range was 0.003–1.25 μM, with repeat analyses of standards reproducible within 10% variation.

In vivo distribution of unconjugated and antibody-conjugated TP

Mice bearing LoVo tumours as xenografts were injected intravenously with TP (20 units/mg) or A5B7-F(ab)$_2$-TP from two separate batches with TP activities of 24 or 7.7 units/mg. The conjugates were prepared using the methodology described previously [11]. This gives a conjugate with the two components linked by a stable thioether linkage. Each mouse received a dose of 1 mg of protein in 100 μl of isotonic saline, equivalent to either 20 units of unconjugated TP or 24 or 7.7 units of A5B7-F(ab)$_2$-TP. Mice were killed and blood, tumour and tissues were removed 0, 12, 24, 36, 48 and 72 h after the administration of 24 units of A5B7-F(ab)$_2$-TP. In separate experiments, blood and tumour were removed 0, 2, 6, 24 and 48 h after the administration of 20 units of unconjugated TP or 7.7 units A5B7-F(ab)$_2$-TP. Plasma was prepared immediately by centrifugation of blood. Tissue and tumour samples were placed on ice for 30 min in lysis buffer, and then homogenized. Samples were microfuged at 13,000*g* for 3 min at room temperature, and the supernatant was collected for analysis by HPLC as described above.

All animal experiments were performed in line with the appropriate Home Office license and conformed to UKCCR guidelines on animal welfare.

Results

Thymidine phosphorylase can prevent thymidine rescue from raltitrexed-induced growth inhibition in vitro

The basal TP activity in the cytosol of the parental HT29 cell line was $0.08 \pm 0.03 \times 10^{-3}$ units/mg, compared with $0.35 \pm 0.03 \times 10^{-3}$ units/mg of protein in the TP transfected HT29 (TP) cell line and $2.54 \pm 0.14 \times 10^{-3}$ units/mg of protein for LoVo cells. Thus, HT29(TP) cells have a fourfold higher TP activity than the parental HT29 cells. The doubling time of both the HT29 parental and TP-transfected cell lines was 32 h and that of the LoVo cell line was 35 h. The concentration of Thd in the dialyzed tissue culture medium was 6.4 nM, whereas the Thd concentration in non-dialysed medium was 24-fold higher at 156 nM. Given the concentrations of Thd required to reverse growth inhibition due to RTX (see below), non-dialyzed medium was used for all subsequent experiments.

After exposure to RTX for three cell-doublings, the IC_{50} values for RTX were 3.0 ± 0.6, 4.0 ± 0.5 and 1.8 ± 0.2 nM in the HT29, HT29(TP) and LoVo cell lines, respectively. The IC_{90} values were 15 ± 1.5 nM in HT29, 15 ± 1.8 nM HT29(TP) and 3.2 ± 0.3 nM in the LoVo cell line. Supplementing the medium of the cell-lines with increasing concentrations of exogenous Thd (0.1–3 μM) progressively reversed the growth inhibitory effects of both IC_{50} and IC_{90} concentrations of RTX (data not shown). Despite the differences in endogenous TP activity among the cell lines, there was no difference in the concentration of Thd necessary to rescue the cells from RTX-induced growth inhibition. Thus, in all three cell lines, 3 μM Thd was required to completely reverse the growth inhibitory effects of an IC_{50} concentration of RTX, while at the IC_{90} concentrations RTX still caused 20% growth inhibition in the presence of 3 μM Thd (data not shown).

Co-incubation with thymidine phosphorylase (25×10^{-3} units/ml) was sufficient to restore RTX-induced growth inhibition at IC_{50} and IC_{90} RTX concentrations in the presence of 3 μM Thd in each of the cell lines (Fig. 1). A concentration of 100 nM TP inhibitor (TPI) was sufficient to block the restoration of sensitivity to RTX induced by TP up to 50×10^{-3} units/ml in the presence of 3 μM Thd (data not shown), demonstrating that the catalytic activity of exogenous TP was necessary for the restoration of sensitivity to RTX in the presence of Thd. A further experiment was performed to determine the effects of RTX, TP and TPI on Thd concentrations in the medium of HT29 and HT29(TP) cells. Figure 2 shows the concentration of

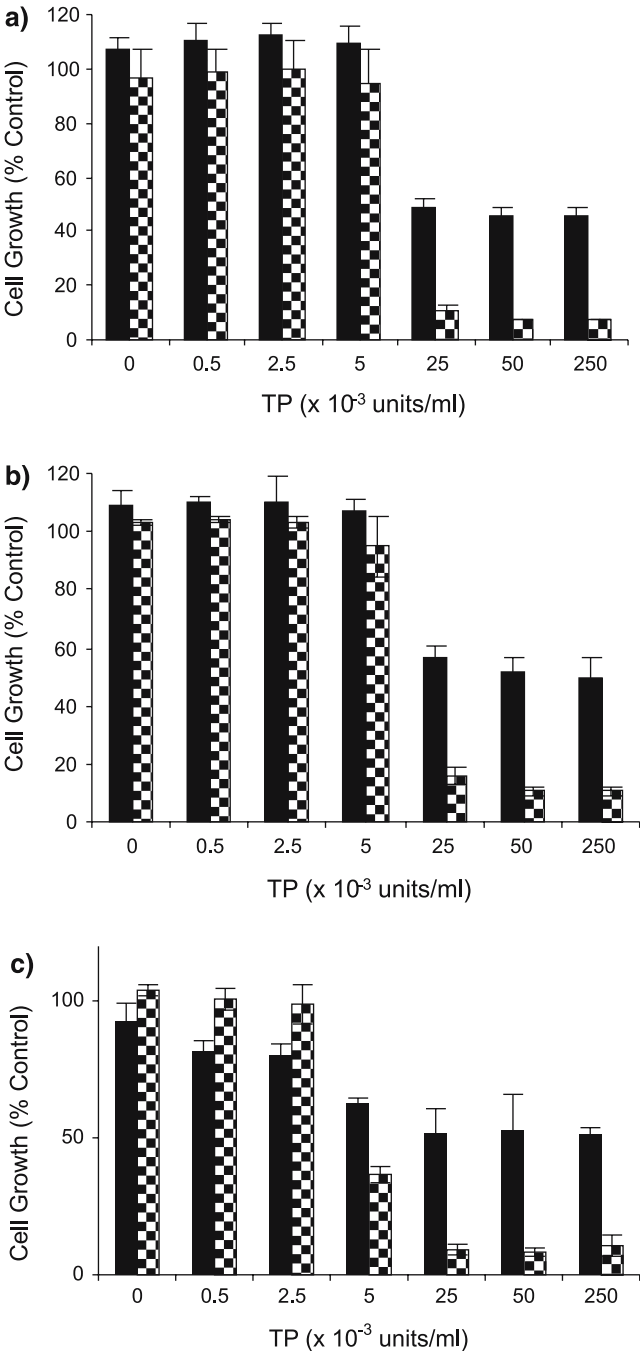

Fig. 1 Growth of **a** HT29, **b** HT29 (TP) and **c** LoVo cells exposed to IC_{50} (*solid bars*) and IC_{90} (*checked bars*) concentrations of RTX and 3 μM Thd in the presence of increasing concentrations of TP. Data are mean ± SD of three separate experiments

Thd in the medium 24 h after supplementing with 3 μM Thd. Co-incubation with RTX at an IC_{90} concentration results in a slight reduction, but addition of 25×10^{-3} units/ml TP substantially depletes extracellular Thd. This effect is blocked by the addition of 100 nM TPI. Of note, there was little or no difference between HT29

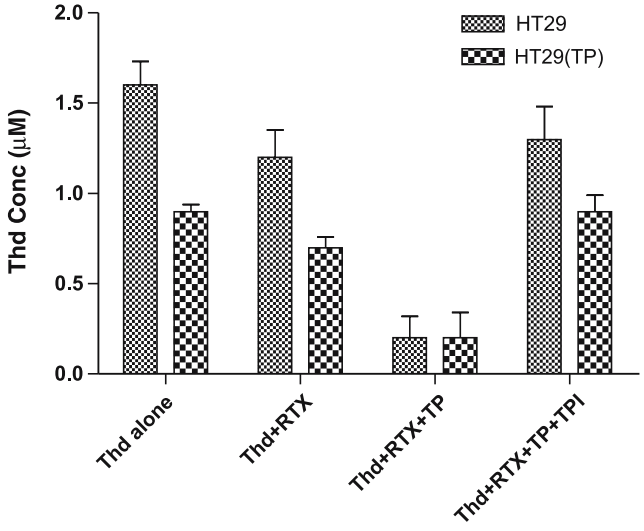

Fig. 2 Thymidine concentrations in the medium of HT29 and HT29(TP)10^6 cells after 24 h of incubation. Medium was supplemented with 3 μM Thd at time zero. Experiments were performed in the absence or presence of RTX at an IC_{90} concentration (15 nM) or TP (25×10^{-3} units/ml) or TPI (100 nM). Values are mean ± SD of three separate experiments

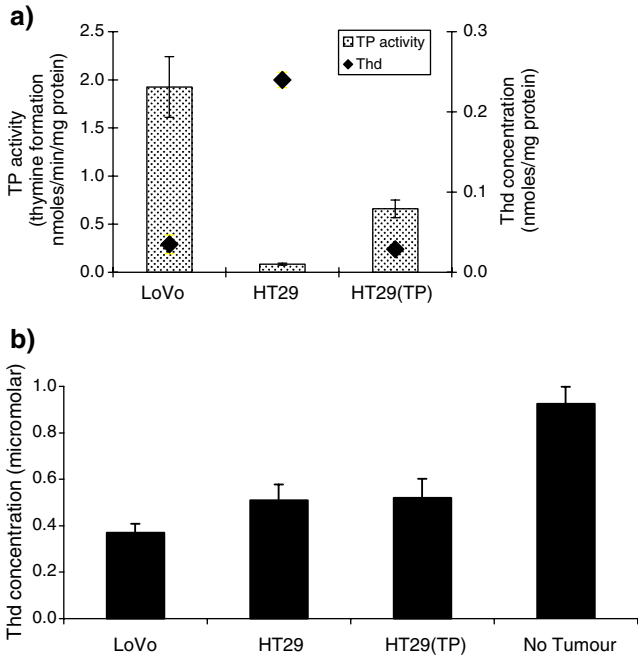

Fig. 3 **a** TP activity (*bars*) and Thd concentration (*diamonds*) in xenografts of a number of human colorectal carcinoma cell lines. **b** Thd concentration in the plasma of mice bearing human colorectal carcinoma xenografts. Data at the mean ± SD of results from five mice

and HT29(TP) cells in the degree of extracellular Thd depletion achieved with exogenous TP.

The impact of xenograft TP and exogenous TP on plasma and tumour Thd concentrations in vivo

LoVo, HT29 and HT29 (TP) cell lines (10^7 cells) were injected subcutaneously into the right flank of female athymic nude mice. Once tumours had reached 1 cm × 1 cm they were removed and separated into two halves for TP and Thd measurements. LoVo and HT29 (TP) tumours, which had relatively high TP activity, had relatively low levels of Thd (Fig. 3a). Conversely, HT29 xenografts with relatively low TP activity had higher levels of Thd. Therefore, the Thd content of the tumours was inversely related to the endogenous TP activity. Furthermore, the rank order and the absolute TP activities in the three cell lines were similar in vitro and in the xenografted tumours.

There was no detectable TP activity in the plasma of control mice or those bearing human tumour xenografts (limit of detection 0.02×10^{-3} units/mg protein). Plasma was taken once xenografts had reached 1 cm × 1 cm, and also from control non-tumour-bearing animals, to determine if the xenografts had any effect on plasma Thd levels. The Thd concentration in the plasma of mice bearing human tumour xenografts was significantly ($P < 0.015$) lower than the concentration in the plasma of control non-tumour-bearing mice (Fig. 3b). Therefore, the presence of subcutaneous tumour xenografts in mice reduces Thd concentrations in the plasma. Thd levels in the plasma of mice bearing LoVo xenografts were significantly ($P < 0.037$) lower than those in mice bearing HT29 parental or TP transfected xenografts. However, there was no significant difference in Thd concentrations in the plasma of mice bearing HT29 parental or TP-transfected cell lines growing as xenografts. LoVo xenografts have at least a 30-fold higher activity of TP relative to HT29 cells, which may explain the lower plasma Thd concentrations in mice bearing LoVo tumours.

Normal tissue TP levels were also studied, and plasma and brain from control animals had no detectable TP activity (less than 0.01×10^{-3} units/mg protein). The TP activity of the other tissues varied; however, TP activity in the tumour was at least fivefold higher than that in any of the other tissues studied (Table 1, $t = 0$). As indicated above, TP activity in the plasma of mice bearing LoVo xenografts was not detectable prior to TP administration; however, 2 h after i.v. administration of 20 units of un-conjugated TP, the concentration of TP in the plasma was 1.3×10^{-3} units/mg of protein (Fig. 4a). This plasma concentration of TP resulted in the reduction of plasma Thd levels to 5 nM from a pre-treatment value of 370 nM. After 6 h the plasma concentration of TP had

Table 1 TP levels after 1 mg of A5B7-F(ab)$_2$-TP conjugate (specific activity 24 units/mg)

Time (h)	TP level ($\times 10^{-3}$ units/mg protein)						
	Colon	Kidney	Liver	Lung	Plasma	Spleen	Tumour
0	0.17 ± 0.09	0.06 ± 0.01	0.38 ± 0.27	0.15 ± 0.02	< 0.02	0.38 ± 0.24	2.14 ± 0.23
12	0.45 ± 0.06	1.20 ± 0.20	0.27 ± 0.11	2.23 ± 0.35	33.40 ± 4.44	0.67 ± 0.19	6.59 ± 0.79
24	0.29 ± 0.05	0.39 ± 0.19	0.53 ± 0.09	0.80 ± 0.35	7.45 ± 3.27	0.35 ± 0.12	8.45 ± 0.99
36	0.17 ± 0.00	0.08 ± 0.04	0.20 ± 0.08	0.67 ± 0.04	7.56 ± 0.50	0.36 ± 0.17	5.46 ± 0.11
48	0.22 ± 0.08	0.08 ± 0.01	0.21 ± 0.09	0.25 ± 0.1	1.48 ± 0.41	0.12 ± 0.02	2.76 ± 0.21
72	0.29 ± 0.10	0.09 ± 0.02	0.62 ± 0.17	0.18 ± 0.04	0.35 ± 0.14	0.17 ± 0.06	2.76 ± 0.54

Female athymic nude mice bearing LoVo xenografts were intravenously injected with 1 mg of A5B7-F(ab′)$_2$-TP conjugate. Colon, kidney, liver, lung, plasma, spleen and LoVo tumour were removed 0, 12, 24, 36, 48 and 72 h after administration of A5B7-F(ab)$_2$-TP conjugate and the TP activity measured using HPLC. Values are mean ± SD of five mice

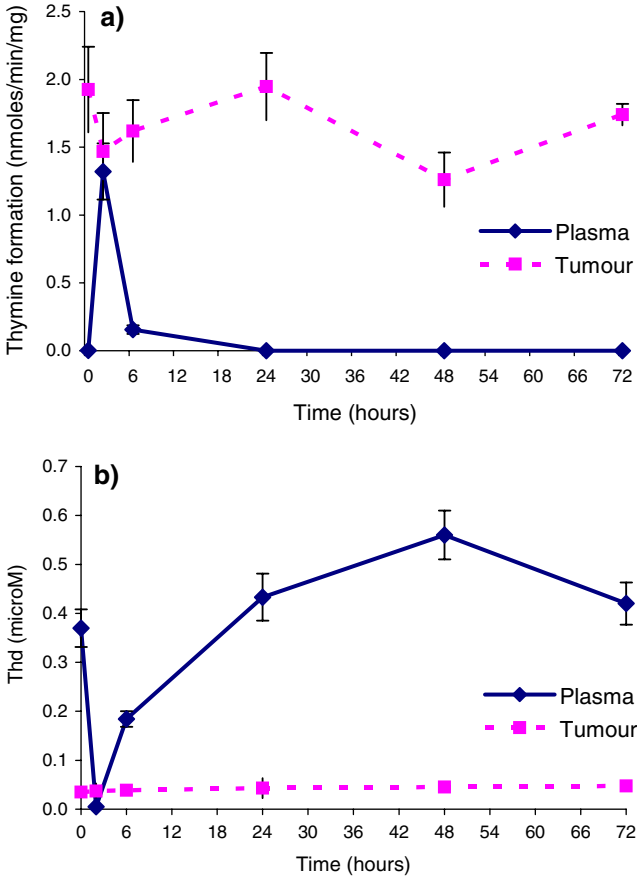

Fig. 4 **a** TP activity and **b** Thd concentration in the plasma (*solid line*) and tumor (*dotted line*) of mice bearing LoVo xenografts after i.v. administration of 1 mg of 20 units/mg unconjugated TP. Data are mean ± SD of results from five mice at each time point

declined to 0.17 × 10^{-3} units/mg, eightfold lower than at 2 h, indicating that un-conjugated TP is rapidly cleared from the plasma in mice. After iv injection of un-conjugated TP in mice bearing HT29 xenografts, plasma and tumour TP activity and Thd levels followed a similar pattern to those seen in LoVo tumour-bearing mice (data not shown), although pretreatment plasma Thd levels were 510 nM in mice bearing HT29 xenografts (Fig. 3b).

In LoVo tumour-bearing animals the decline in plasma TP activity 2–6 h after un-conjugated TP administration was accompanied by recovery of plasma Thd levels (Fig. 4b). Twenty-four hours after administration of TP there was no detectable TP in the plasma (Fig. 4a) and the Thd levels had returned to pretreatment values (Fig. 4b). There was a small degree of rebound in the Thd levels at 48 h, which may indicate compensation for the initial loss of Thd. Notably, TP activity and Thd concentrations in the LoVo tumours were not markedly altered by the administration of 20 units of unconjugated TP (Fig. 4a, b), and the same result was obtained in mice bearing HT29 tumours (data not shown). Thus un-conjugated TP does not accumulate in tumour tissue or influence tumour Thd levels in the mouse LoVo or HT29 xenograft models.

TP conjugated to A5B7 F(ab)2 does not selectively deplete tumour Thd

In an attempt to target TP to tumour tissue, two experiments were performed in which TP conjugated to the A5B7 F(ab)$_2$ antibody fragment was administered. The first batch of conjugate used had a specific activity of 24 units/mg, while the specific activity of that used in the second experiment was 7.7 units/mg.

The activity of TP in tissues and plasma after treatment with 1 mg of 24 units/mg A5B7-F(ab)$_2$-TP conjugate are shown in Table 1. The TP activity in the plasma 12 h after the administration of the conjugate was 33 × 10^{-3} units/mg protein, which decreased fourfold to 7.4 × 10^{-3} units/mg protein by 24 h, indicating that conjugated TP is cleared more slowly than un-conjugated TP. The TP activity in the plasma was 0.35 × 10^{-3} units/mg protein 72 h after i.v. administration of 1 mg of the conjugate. The level of TP in the brain remained < 0.02 × 10^{-3} units/mg protein (data

not shown) throughout, and activity in the colon, liver and spleen remained below 1×10^{-3} units/mg protein, with at most a twofold increase over pretreatment concentrations after conjugate administration. Although there was an increase in TP activity in the kidney and the lungs at 12 h, TP activity in the normal tissues studied did not exceed the pretreatment levels in the LoVo tumour at any time point.

TP activity in the tumour was, relative to pretreatment values, significantly ($P < 0.0008$) elevated to 6.6 (threefold elevation), 8.4 (fourfold elevation) and 5.5 (2.5-fold elevation) $\times 10^{-3}$ units/mg protein 12, 24 and 36 h, respectively, after administration of 24 units of the conjugate (Table 1). At 48 h the TP activity in the tumour was approaching pretreatment values, but was still significantly higher at 2.8×10^{-3} units/mg protein compared to the pretreatment value of 2.1×10^{-3} units/mg protein ($P = 0.03$). Seventy-two hours after administration of the conjugate, TP activity in the tumour was not significantly ($P = 0.15$) different from pretreatment levels. Forty-eight and 72 h after administration of the conjugate the TP activity in the plasma was lower than the TP activity in the tumour, with tumour/plasma TP activity ratios of 1.9 and 7.9, respectively. Due to TP levels in plasma prior to treatment being undetectable, the pretreatment tumour/plasma TP activity ratio could only be estimated at > 100. Thus although the levels of TP in the tumour at 48 and 72 h were higher than those in the plasma, the tumour/plasma differential was far less than in the pretreatment samples.

To determine the effect of the A5B7-F(ab)$_2$-TP conjugate on Thd concentrations in the plasma and tumour of mice bearing LoVo xenografts, a conjugate with a specific activity of 7.7 units/mg were injected iv at a dose of 1 mg/mouse. After A5B7-F(ab)$_2$-TP conjugate administration, the TP activity in the plasma was increased at 2 and 6 h (Fig. 5a), at which time point plasma Thd levels were decreased from 370 to 20 nM (Fig. 5b). Twenty-four hours after administration of the conjugate, the TP activity was 16-fold lower than the activity after 6 h, and this reduction of TP in the plasma was associated with a recovery of Thd concentrations to pretreatment levels. At 48 h, the TP activity in the plasma and tumour had declined to values near to those seen in untreated mice (i.e. $< 0.01 \times 10^{-3}$ units/mg protein), with the concentration of Thd in the plasma increased further, such that it exceeded pretreatment levels by twofold. With the lower specific activity 7.7 units/mg conjugate, tumour TP levels were elevated by no more than a factor of 2 and Thd concentrations were not reduced relative to pretreatment values (Fig. 5).

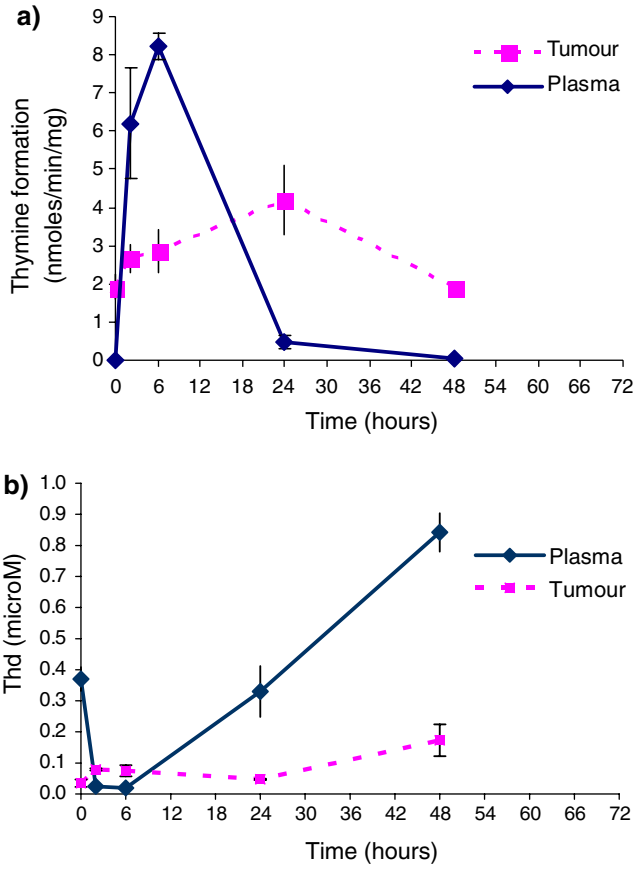

Fig. 5 **a** TP activity and **b** Thd concentration in the plasma (*solid line*) and tumor (*dotted line*) of mice bearing LoVo xenografts after i.v. administration of 1 mg of 7.7 units/mg A5B7 F(ab′)$_2$-TP conjugate. Data are mean ± SD of results from three mice at each time point

Discussion

Thymidylate synthase inhibitors have been extensively investigated in cancer treatment [6] and, to overcome resistance due to thymidine salvage, TS inhibitors have been combined with inhibitors of TK [17], nucleoside transport [22] and TP [14]. In the current studies, TP was used with RTX and the ability of TP to prevent Thd salvage evaluated in vitro and in vivo.

Consistent with previous studies in colorectal carcinoma cell lines, RTX caused 90% growth inhibition at low nanomolar concentrations in the HT29, HT29(TP) and LoVo cells. Despite the higher TP activity in the HT29(TP) cells, there was no difference in sensitivity to RTX. This is because, in the absence of supplementary Thd, there is insufficient Thd present in the medium to affect sensitivity to TS inhibition. Blocking de novo thymidylate synthesis causes cells to increase the uptake and incorporation of

extracellular thymidine for thymidylate formation. It has previously been reported in the MGH-U1 bladder cell line that the activity of TK and the expression of the es or ENT1 nucleoside transporter increased 4.5-fold and 39-fold, respectively, in the presence of 10 nM RTX [18], indicating increased dependence on Thd salvage once TS is inhibited. Thus, although Thd salvage occurs in the absence of a TS inhibitor, cells become more dependent on the salvage pathway for the formation of dTMP when TS is inhibited. As shown in the current study, Thd alone rescues cells from RTX-induced growth inhibition, confirming that TS is the sole locus of action for the drug in the cell lines studied. This result is consistent with previous studies showing that, in the presence of 10 μM Thd, GI_{50} concentrations for RTX in the HT29, LoVo and SW620 were increased over 12,000-fold [9]. Sensitivity to RTX in the presence of Thd was restored by TP, consistent with data showing the depletion of Thd in the medium, and again the ability of TP to deplete Thd and increase sensitivity to RTX has been reported previously [14].

These in vitro data suggest that TP can sensitize tumour cells to RTX, even in the presence of high concentrations of extracellular Thd. In vivo experiments were therefore performed to investigate the potential of antibody-mediated delivery of TP to tumour tissue in a xenograft model, as measured by effects on plasma and tumour Thd and TP levels. TP activity in the human colorectal carcinoma cell lines grown as xenografts in vivo was the same as that determined in vitro, and there was no TP activity in the plasma of the mice used in these experiments regardless of whether, or not, they were bearing xenografts. Implantation of either LoVo or HT29 tumour xenografts decreased the Thd concentration in the plasma from 0.9 to 0.4–0.6 μM. This reduction in Thd concentration in the plasma may be due to the increased salvage of Thd by the tumour, as salvage pathway enzyme activity is reported to be increased in tumour cells relative to normal tissues [13]. Furthermore, the tumour Thd concentration was related to the tumour TP activity and thus the endogenous tumour TP level can influence both the plasma and tumour Thd concentration in xenograft models.

Following the administration of un-conjugated TP there was a substantial, but transient, decline in plasma Thd concentrations. However, the TP activity and Thd concentrations returned to basal levels 24 h after administration of unconjugated TP. In contrast, A5B7 $F(ab)_2$ conjugated TP persisted in the plasma for longer, potentially allowing the conjugate to accumulate within tumour tissue.

Following the administration of A5B7-$F(ab)_2$-TP conjugate, with a specific activity of 24 or 7.7 units/mg, TP activity in the tumour was increased fourfold or twofold, respectively, at 24 h. TP activity in the tumour was at least threefold greater than that in any of the other tissues, whereas after the administration of un-conjugated TP there was no tumour localization. The LoVo cell line used for the xenograft model expresses high levels of CEA (56 ng of CEA per 10^6 cells, data from ATCC website), the target of the A5B7 antibody, consistent with the conjugate localization observed.

After administration of A5B7-$F(ab)_2$-TP conjugate there was a greater level of TP in the tumour after 24 h than in the plasma, but the differential was not as great as previously seen when the $F(ab)_2$ fragment alone was used in the same tumour model [2]. However, the difference in the size of the conjugate (145 kDa if monomer and 190 kDa if dimer, supplied in a 2:1 ratio) compared with the $F(ab)_2$ fragment (100 kDa) may explain the difference in the retention in the plasma. The maximum TP concentration in plasma and tumour was observed 12 and 24 h, respectively, after administration of the A5B7-$F(ab)_2$-TP conjugate. However, at no time point were the tumour:plasma TP ratios higher than in pre-treatment samples. In vivo preclinical experiments with radiolabelled A5B7 $F(ab)_2$ fragment conjugated to carboxypeptidase (CPG2) have shown that with an optimised conjugate a maximum of 3% of the injected dose per gram of tissue could be delivered to LoVo tumours 24 h after administration [2]. In the current experiment, using A5B7-$F(ab)_2$-TP conjugates with two different specific activities, 2% of the injected TP activity/gram of tissue had accumulated in the LoVo tumour 24 h after injection. Therefore the A5B7-$F(ab)_2$-TP conjugate performed comparably to the previously characterized CPG2 constructs used in antibody directed enzyme pro-drug therapy.

To evaluate the likely gain in therapeutic effect for RTX following depletion of Thd by TP, the innate tumour expression of TP needs to be considered. Expression of TP may be higher in tumours compared to corresponding normal tissues [23]. Indeed, high TP expression or activity has been associated with a favourable response to 5-FU-based chemotherapy [4, 10], in keeping with the role of TP in the activation of 5-FU. In other circumstances, the pro-angiogenic activity of TP has resulted in an association with poor outcome when overexpressed in tumours [12]. Activity of TP in colorectal tumours has been reported to range from 0.5 to 10.7 (mean 3.3) $\times 10^{-3}$ units/mg protein [24]. Thus, for some tumours the additional 6×10^{-3} units/mg protein delivered by the A5B7-TP conjugate would be significant, but for others would

not result in a major increase in TP activity. It should also be noted that in the current study Thd concentrations were suppressed in the HT(29)TP cell line, which has a TP activity of only 0.7 nmol/min/mg. Thus, the gain from further selective delivery of TP to the tumour may be limited. As observed in our in vivo experiments, although delivery of TP to the tumour was achieved, this did not result in any significant depletion of Thd in the tumour, perhaps due to the high intrinsic TP activity of the Lovo cell line.

In conclusion, this study has demonstrated that Thd alone can rescue human colorectal carcinoma cells from cell growth inhibition induced by RTX, and that exogenous TP can restore sensitivity by metabolising extracellular Thd. In vivo experiments have shown that, although conjugation of TP to the A5B7 $F(ab)_2$ fragment delivers TP to the tumour, there is no enhanced or selective depletion of Thd in the tumour. Therefore no therapeutic advantage would be expected for RTX in combination with the A5B7-$F(ab)_2$-TP conjugate in the LoVo xenograft model compared to RTX alone.

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