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ABSTRACT

The enzyme thymidine phosphorylase (TP) is important for activation of capecitabine and 5-fluorouracil. Assessment of TP phenotype might be suitable for identification of patients at risk of fluoropyrimidine-induced toxicity. In this paper, we describe the development and validation an assay for TP activity in peripheral blood mononuclear cells (PBMCs). The assay was based on *ex vivo* conversion of the TP substrate thymidine to thymine. The amount of thymine formed was determined by high-performance liquid chromatography – ultraviolet detection (HPLC-UV) with 5-bromouracil as internal standard. Lymphocytes and monocytes were purified from isolated PBMCs to examine cell-specific TP activity. TP activity in PBMCs demonstrated Michaelis-Menten kinetics. The lower limit of quantification was 2.3 μg PBMC protein and assay linearity was demonstrated up to 22.7 μg PBMC protein. Within-day and between-day precisions were ≤9.2% and ≤6.0%, respectively. Adequate stability TP activity was demonstrated after long-term storage of PBMC dry pellets and lysates at −80 °C. In monocytes, TP activity was approximately 3 times higher than in lymphocytes. Clinical applicability was demonstrated in samples that were collected from five cancer patients. A simple, precise and sensitive HPLC-UV assay for quantification of TP activity in PBMCs was developed that can be applied for clinical research.

1. Introduction

Capecitabine is an orally available pre-prodrug of 5-fluorouracil (5-FU) that is used for treatment of colorectal, gastric and breast cancer. After administration, capecitabine is rapidly absorbed and enzymatically

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converted to subsequently 5′-deoxy-5-fluorocytidine (dFCR) and 5′-deoxy-5-fluoro-uridine (dFUR).[1–5] Conversion of dFUR to 5-FU is catalyzed by thymidine phosphorylase (TP) and uridine phosphorylase (UP).[6,7] Because of relatively high TP and UP expression, formation of 5-FU preferentially occurs within tumor and liver tissue.[4,8,9] Approximately 80% of 5-FU is catabolized to inactive metabolites by the enzyme dihydropyrimidine dehydrogenase (DPD) and about 1-3% of 5-FU is intracellularly anabolized to active metabolites. The metabolite 5-fluoro-2′-deoxyuridine-5′-mono-phosphate (FdUMP) possesses the highest anticancer potency. This metabolite inhibits the enzyme thymidylate synthase (TS), which leads to disrupted DNA synthesis and cell death.[10] Formation of FdUMP also depends on TP, since TP catalyzes intracellular conversion of 5-FU to 5-fluoro-2-deoxyuridine (FdUrd), which, in turn, is converted to FdUMP by thymidine kinase. The activation pathway of capecitabine is illustrated in more detail in Figure 1.

About 10-30% of the patients treated with capecitabine develop severe, sometimes even lethal, toxicity.[11] Recent studies by us and others showed that some single nucleotide polymorphisms (SNPs) in the gene encoding DPD (*DPYD*) are associated with capecitabine-induced toxicity.[12,13] Genotyping for relevant SNPs in *DPYD* alone, however, has limited sensitivity for identification of patients at risk of severe toxicity.[13]

Recently, a single nucleotide mutation (*1412C > T,* rs11479) in the gene for TP (*TYMP*) was found to be associated with relatively high expression of TP and capecitabine-induced diarrhea and hand-foot syndrome in patients.[14,15] *In vitro* studies have demonstrated that the cytotoxic effects of the TP substrate dFUR are increased after upregulation of TP.[16]. Furthermore, upregulation of TP has been found in tumor tissue of patients after treatment with docetaxel, adriamycin and epirubicin.[17–19] Studies of xenograft models and *in vitro* experiments also demonstrated TP induction after exposure to vorinostat, vinorelbine, lidamycin and X-ray irradiation.[20–23] Based on these findings, it seems likely that the availability of a marker for the TP phenotype could attribute to the identification of patients at risk of capecitabine-induced toxicity. Since TP activity seems to be affected by other treatments, the ideal TP phenotype marker should allow for longitudinal assessments of TP activity.

Peripheral blood mononuclear cells (PBMCs) are extensively used as a source of surrogate tissue for quantification of a phenotype marker.[6,7] Collection of PBMCs is minimally invasive and can be repeated at several time points in order to assess treatment effects. A method for phenotyping TP activity in total leukocytes has previously been described.[24] This method is based on quantification of the amount of thymine formed after *ex vivo* incubation with the TP substrate thymidine. Importantly,
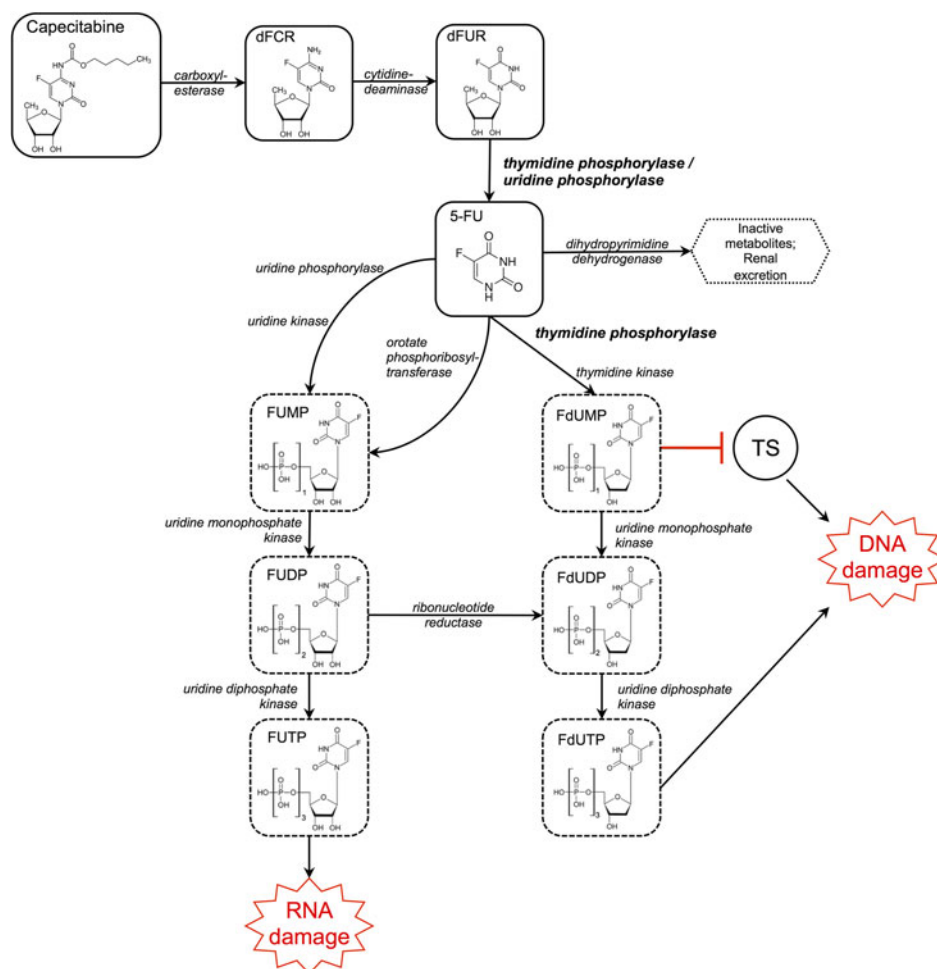
Figure 1. Activation pathway of capecitabine. The role of thymidine phosphorylase is highlighted in bold. Abbreviations: dFCR, 5'-deoxy-5-fluorocytidine; dFUR, 5'-deoxy-5-fluorouridine; 5-FU, 5-fluorouracil, FdUMP, 5-fluoro-2'-deoxyuridine-5'-monophosphate; TS, thymidylate synthase; FUMP, 5-fluorouridine-5'-monophosphate; FUDP, 5-fluorouridine-5'-diphosphate; FUTP, 5-fluorouridine-5'-triphosphate; FdUDP, 5-fluoro-2'-deoxyuridine-5'-diphosphate; FdUTP, 5-fluoro-2'-deoxy-5'-triphosphate.

TP activity significantly differed among leukocyte subpopulations.[25] The PBMC population mainly consists of lymphocytes and a small percentage of monocytes. Therefore, the PBMC population is more homogenous than the total leukocyte population and is possibly less distorted by alterations in relative sample composition.

The primary objective was to develop and validate a simple assay for quantification of TP activity in PBMCs (TPA$_{pbmc}$). In addition, we explored the TP activity in the purified lymphocytes and monocytes.

2. Materials and methods

2.1. Chemicals

Thymidine, thymine, 5-bromouracil (5-BU), dithiothreitol (DTT), potassium dihydrogenphosphate (KH_2PO_4), dipotassium hydrogenphosphate (K_2HPO_4), high-performance liquid chromatography (HPLC)-grade methanol, bovine serum albumin (BSA) and Hoechst33258 were purchased from Sigma (St. Louis, MO, USA). The water used for the experiments was Milli-Q grade (Millipore, Billerica, MA, USA). Ficoll-paque[tm] PLUS was obtained from General Electric Healthcare (Little Chalfont, UK). Phosphate buffered saline (PBS) was purchased from Gibco BRL (Gaithersburg, MD, USA). Perm/Wash[tm] was obtained from Becton Dickinson (Heidelberg, Germany) and formaldehyde was purchased from Merck (Darmstadt, Germany). Magnetic antibody cell sorting (MACS) columns, anti-CD14 microbeads, anti-CD45 fluorescein isothiocyanate (FITC), anti-CD14 allophycocyanin (APC) were obtained from Miltenyi (Bergisch Gladbach, Germany).

2.2. Isolation of PBMCs

Heparinized blood (8 mL) was mixed with an equal volume PBS. Isolation of PBMCs was achieved using Ficoll density gradient centrifugation at 720g for 20 min at room temperature. The leukocyte layer containing PBMCs was transferred to a clean 50 mL tube, washed with PBS and centrifuged at 1000g for 10 min at 4 °C. Then, PBMCs were washed twice with PBS, centrifuged at 500g for 10 min at 4 °C and transferred to a 1.5 mL cryovial. After centrifugation, supernatant was removed and the PBMCs were snap-frozen in liquid nitrogen and stored at −80 °C until further processing.

2.3. Sample preparation for TP activity measurement

After defrosting, PBMCs were resuspended in 300 μL assay buffer (35 mM potassium phosphate, 1 mM DTT; pH 7.4) and divided into three 100 μL aliquots that were independently processed. Samples were sonicated for 15 pulses using a Branson 250 tip sonicator (Branson, Danbury, USA) that was set on program 3 and 50% duty. PBMC cytosolic lysate was isolated after centrifugation at 11,000g for 20 min at 4 °C. Protein concentration was determined using the Bradford assay (Bio-Rad protein assay kit, Bio-Rad, Hercules, CA, USA).[26] The amount of hemoglobin contamination in PBMC cytosolic lysate was quantified using a validated spectrophometrical method and subtracted from the total PBMC cytosolic protein.[27]

2.4. TP enzyme activity assay

The assay was based on a method for TP activity of the total leukocyte population, with modifications.[24] TPA_{pbmc} was expressed by the amount of thymine formed after incubation of the TP substrate thymidine. Incubation started when PBMC cytosolic lysate was added to 2 mM thymidine in assay buffer in a total reaction volume of 500 μL. Three negative control samples consisting of 2 mM thymidine in assay buffer were freshly prepared for each run. Samples were incubated for 1 hour at 37 °C. Directly after incubation, 50 μL of the ice-cold internal standard solution (100 μg/mL 5-BU in Milli-Q water) was added and the reaction was terminated by placing the samples on a heat block for 4 min at 100 °C. After centrifugation at 11,000g for 5 minutes at 4 °C, clear supernatant was transferred to a glass vials and a volume of 60 μL was injected into the HPLC system coupled with ultraviolet detection (HPLC-UV).

2.5. HPLC-UV analysis

Thymine and 5-BU concentrations were quantified using an UltiMate 3000 HPLC-UV system (Dionex, Sunnyville, CA, USA). Chromatographic separation was achieved on an Interchrom C_{18} column (150 x 4.6 mm ID, particle size 5 μm; Interchim, Montluçon Cedex, France). The autosampler and the column were at room temperature. Eluent A consisted of 50 mM KH_2PO_4 (pH 4.5) and 1.0% (v/v) methanol in water and eluent B consisted of 50 mM KH_2PO_4 (pH 4.5) and 40% (v/v) methanol in water. The following gradient was used: 20% B from 0-8 min, 20-100% B from 8-9 min, 100% B from 9-14 min, 20% B from 14-20 min. The flow rate was 0.8 mL/min. Thymine and 5-BU were quantified at 265 nm. An external calibration curve was prepared in duplicate with thymine concentrations ranging from 0.76 to 500 μM. The amount of thymine in the negative control samples was subtracted from the amount of thymine in study samples. Chromeleon software (Dionex, Sunnyville, CA, USA; version 6.8) was used to control the HPLC-UV system and for data processing.

2.6. Method validation

2.6.1. TP enzyme kinetics
The influence of thymidine concentration on TPA_{pbmc} was investigated by running the assay with 0.98, 1.95, 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 μM thymidine. For all reactions, the amount of PBMC cytosolic protein was 10 μg. Non-linear regression, using the Michealis-Menten equation, was performed to determine the V_{max} and K_m. A linearized model of Michealis-Menten, the Eadie-Hofstee model, was used for data visualization.

Assay linearity and the lower limit of quantification (LLoQ) were determined by running the assay with 0.9, 2.3, 4.5, 9.1, 18.2 and 22.7 µg of PBMC cytosolic protein. Linear regression of TPA_{pbmc} versus protein input was performed. TPA_{pbmc} values were back-calculated from the regression line and deviations from the measured TP activities were determined. Back-calculated TPA_{pbmc} should not deviate from the observed TPA_{pbmc} by more than ±20% at the LLoQ level and ±15% for higher protein input levels. Assay precision of ≤20% at the LLoQ level and ≤15% at the higher cytosolic protein input levels was considered acceptable. The limit of detection (LoD) of the TP method was defined as the average thymine background (µM) signal from three negative control samples plus three times the standard deviation.

Time dependency of the TP reaction was assessed in samples that were incubated for 15, 30, 60, 90, 120 and 210 minutes at 37 °C using 12.5 µg PBMC cytosolic protein. The effect of temperature on TPA_{pbmc} was assessed by running the assay at 0, 25, 37, 50, 60 and 70 °C with 10 µg of PBMC cytosolic protein. Samples were equilibrated for 15 minutes at the different temperatures before thymidine was added. Kinetics of TP was assessed using pooled PBMCs from three healthy volunteers.

2.6.2. Within-day and between-day precision

Within-day precision (WDP) and between-day precision (BDP) were determined from quantification of TPA_{pbmc} in 5 consecutive analytical runs. PBMCs of one healthy volunteer were aliquoted in 5 cryovials and stored at -80 °C. On each day, one sample was thawed, prepared and analyzed in triplicate using 5 and 15 µg of PBMC cytosolic protein for the enzymatic reaction. One-way analysis of variance (ANOVA) with run day as classification variable was performed in order to calculate WDP and BDP [26]. Assay precision was considered acceptable in case WDP and BDP were <15%.

2.6.3. Specificity

Specificity was determined using three batches of blank PBMCs. Samples with 20 µg of PBMC cytosolic protein were subsequently spiked with 2.5, 12.5 and 50 µM of thymine. Deviations from the nominal thymine concentrations were determined.

2.6.4. Stability

The stability of TPA_{pbmc} was examined after storage of whole blood for 4 and 24 hours at room temperature and for 4 hours on ice-water. Long-term stability of TPA_{pbmc} was assessed after storage of PBMC dry pellets and

PBMC cytosolic protein lysates for 60 days at $-80\,°C$. The stability of processed samples in the HPLC autosampler was assessed after 24 hours of storage at room temperature. Assay buffer and thymidine (50 mM) stock solution were stored for 2 months at $-20\,°C$, before the effect on TPA_{pbmc} was determined. Stability of TPA_{pbmc} was considered acceptable if 85-115% of the initial activity was obtained.

2.7. Clinical applicability

Clinical applicability of the developed assay was assessed by quantification of TPA_{pbmc} in patient samples. The samples were collected from cancer patients who participated in a phase I clinical study of chronomodulated capecitabine therapy (http://www.trialregister.nl, study identifier: NTR4639). From each patient, 4 mL of blood was drawn within 3 days prior to treatment with capecitabine (screening sample), at the 7th day of capecitabine treatment and at the end of treatment. Samples were collected at 9:00 h to avoid possible interference of circadian variability in enzyme kinetics. Immediately after blood collection, PBMCs were isolated and stored as dry pellets at $-80\,°C$ until further processing. The study was approved by the Ethics Committee of the Antoni van Leeuwenhoek Hospital, Amsterdam, The Netherlands.

2.8. TP activity PBMC subpopulations

2.8.1. Isolation of monocytes and lymphocytes

TP activities in the monocyte and lymphocyte subpopulations were explored in samples that were obtained from six healthy volunteers. PBMCs were isolated from 24 mL of heparinized blood using Ficoll density gradient. After washing the PBMCs three times with PBS, the cells were resuspended in 1 mL of beads buffer (BB) that consisted of 0.5% BSA and 2 mM EDTA in PBS. A total of 15 x 10^6 PBMCs were used for separation and isolation of the monocyte and lymphocyte subpopulations. The remaining PBMCs were used for assessment of TP activity in the total PBMC population. A volume of 8 μL of anti-CD14 microbeads, which was used for positive selection of monocytes, was added to the 15 x 10^6 PBMCs in a total volume of 200 μL and incubated for 30 minutes at room temperature. After incubation, PBMCs were washed three times with 1 mL BB in order to remove unbound anti-CD14 microbeads. The PBMCs were resuspended in 500 μL BB and the cell suspension was loaded on a MACS column for magnetic enrichment. The lymphocytes, which are CD14-negative, were collected by rinsing the MACS column three times with 500 μL BB. After removal from the magnetic field, the column was flushed twice with

1 mL of BB in order to elute the monocytes. The samples were centrifuged at 1000g for 4 min at 4 °C. After centrifugation, the supernatant was removed, leaving 100 μL buffer on the pellets. Sample purity was assessed by analyzing 5 μL of each sample by flow cytometry. The isolated monocytes, lymphocytes and the sample containing the total PBMC population were washed twice with PBS and once with TP assay buffer, before the TP activity was assessed. Differences in TP activity among monocytes, lymphocytes and total PBMC populations were assessed by one-way analysis of variance and Tukey's post-hoc tests.

2.8.2. Flow cytometry analysis

Fluorescence-activated cell sorting (FACS) analysis was performed to assess the amount of monocytes and lymphocytes in the processed samples. From each sample, a 5 μL aliquot was fixed in 1 mL of 2% formaldehyde in BB (v/v) for 15 minutes at room temperature. The samples were centrifuged for 4 minutes at 1000g at 4 °C and washed twice with 1 mL BB. After discarding the supernatant, 1 mL of Perm/Wash[tm] was added and the samples were inverted 10 times, followed by centrifugation at 1000g for 4 minutes at 4 °C. Supernatants were carefully removed, leaving 100 μL on the cell pellets. The cells were stained with 3 μL anti-CD45-FITC, 1.5 μL anti-CD14-APC and Hoechst33258. After incubation for 1 hour at room temperature, the cells were washed twice with 1 mL Perm/Wash[tm]. Samples were centrifuged at 1000g for 4 minutes at 4 °C and supernatants were removed, leaving 100 μL on the cell pellets. A volume of 300 μL BB was added. Flow cytometry was performed with a CyAn ADP Analyzer (Beckman Coulter, Brea, CA, USA). Summit software (Dakota Cytomation, Fort Collins, CO, USA; version 4.3) was used for data analysis.

2.9. Statistics

Statistical analyses were performed using Prism 6 (GraphPad, La Jolla, CA, USA). *P*-values ≤0.05 were considered statistically significant.

3. Results

3.1. Method development

Quantification of TPA$_{pbmc}$ was achieved by determining the amount of thymine formed after incubation of thymidine in the presence of PBMC cytosolic protein and the co-factor phosphate. We used a previously optimized method for isolation of PBMC cytosolic lysate.[26,28] For the current method, we adapted the same procedures for processing PBMCs.

In order to quantify DPD activity in PBMCs, we previously established a HPLC method for chromatographic separation of thymine and dihydrothymine.[28] The same HPLC column was used for the current assay. We used 5-BU as an internal standard because this substance closely resembles the physicochemical properties of thymine. The proportion of eluent B was titrated up to a level that allowed for adequate separation of thymine and 5-BU. Typical retention times for thymine and 5-BU were 6.6 and 7.8 min, respectively. Thymidine eluted after 12.1 min and was not quantified during the HPLC-UV analysis, since the reduction in thymidine levels after incubation with PBMC cytosolic lysate was relatively small and could not be accurately quantified. The chromatogram of a sample that was processed with 20 µg of PBMC cytosolic protein without thymidine and internal standard did not reveal interfering peaks at the retention times of thymine and 5-BU (Figure 2A). A small peak was observed at the expected retention time of thymine in a sample with heat-treated PBMC lysate (20 µg) that was spiked with 2 mM thymidine (Figure 2B). In addition, this small peak was also observed at the expected retention time of thymine in a sample that only contained 2 mM thymidine in assay buffer (Figure 2C). There was no thymine peak in the sample that only contained heat-treated PBMC cytosolic protein and 5-BU (Figure 2D). The chromatogram of a representative study sample that was incubated with 15 µg PBMC cytosolic protein resulted in a relatively large increase of the thymine peak (Figure 2E). Because of the background signal in control samples of 2 mM thymidine in assay buffer and heat-treated PBMC lysate (Figure 2B-C), three negative control samples were freshly prepared for each analytical run. The average thymine concentration in the background samples was subtracted from the thymine levels in the study samples.

3.2. Method validation

3.2.1. TP enzyme kinetics

The kinetics of TPA_{pbmc} with varying thymidine concentrations is illustrated in Figure 3. TPA_{pbmc} clearly demonstrated Michaelis-Menten kinetics. The estimated V_{max} and K_m were 1278 nmol/mg/h (95% confidence interval (CI) = 1255-1301 nmol/mg/h) and 78.1 µM (95% CI = 73.2-83.0 µM), respectively.

The amount of thymine increased linearly between 2.3 and 22.7 µg of PBMC cytosolic protein that was used for the enzymatic reaction (Figure 4A). At the LLoQ level of 2.3 µg protein input level, the average (± s.d.) thymine concentration was 6.78 ± 0.13 µM and highly exceeded thymine levels in blank control samples (1.61 ± 0.02 µM). The assay LoD was 1.67 µM. Assay accuracy and precision were 94.4% and 2.8% at the
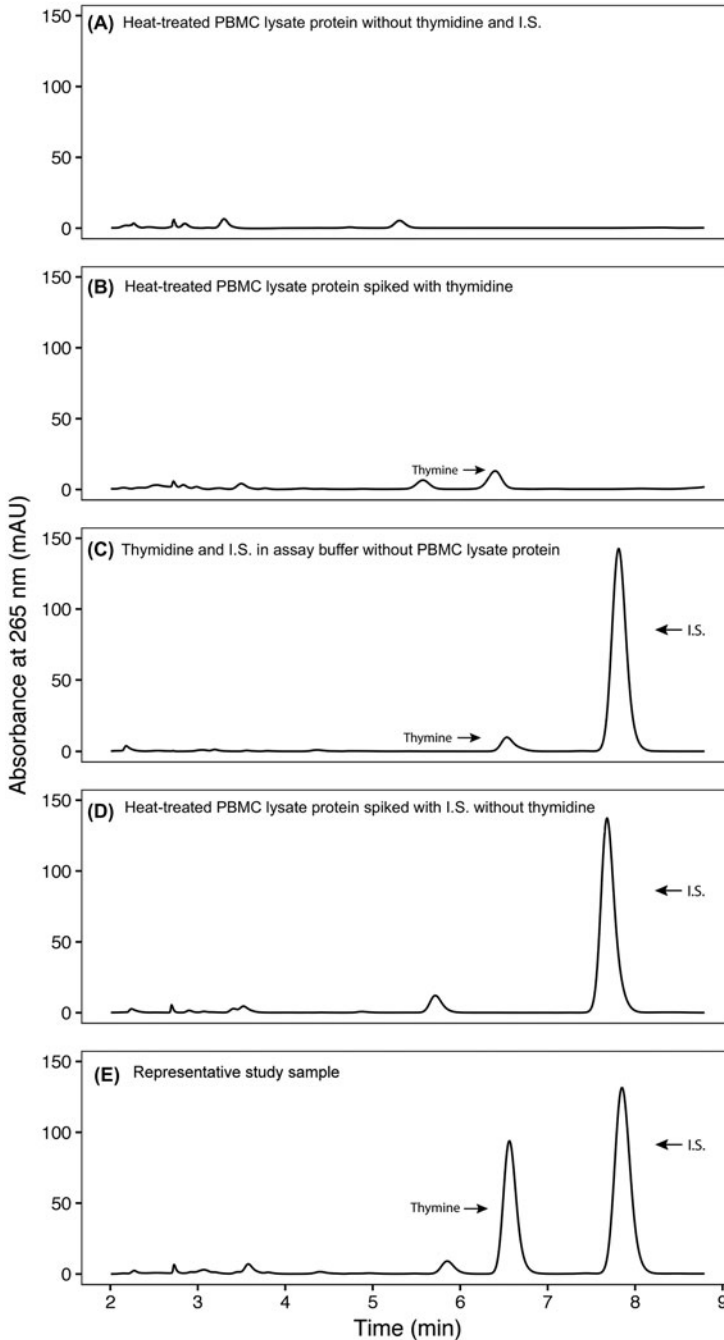
Figure 2. Representative chromatograms of thymine and the internal standard 5-bromouracil in A) a negative control sample of 20 μg PBMC cytosolic protein in assay buffer B) a negative control sample of 20 μg of PBMC protein spiked with thymidine C) a negative control sample of 2 mM thymidine and internal standard in assay buffer D) a control sample of 20 μg PBMC cytosolic protein and internal standard E) a study sample incubated with 15 μg of PBMC cytosolic protein and 2 mM thymidine in assay buffer. Abbreviations: PBMC, peripheral blood mononuclear cells; I.S., internal standard.
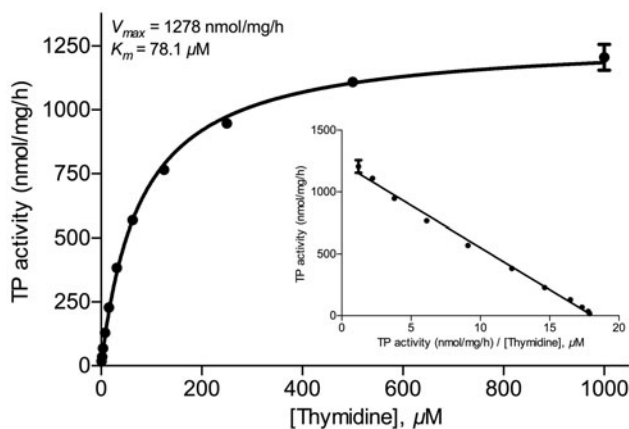
Figure 3. The effect of thymidine concentration on thymidine phosphorylase activity in peripheral blood mononuclear cells that were pooled from 3 volunteers. Data are visualized by a Michaelis-Menten and linearized (Eadie-Hofstee) plot (insert). Results are expressed by the average ± s.d. of three samples. Abbreviations: TP, thymidine phosphorylase; V_{max}, maximum enzyme velocity; K_m, Michaelis-Menten constant.

LLoQ level. At protein input levels of 4.5, 9.1, 18.2 and 22.7 μg, the accuracy and precision ranged between 98.8-107.5% and 1.6-3.6%, respectively.

There was a linear association between the amount of thymine formed and the duration of incubation (Figure 4B). TPA_{pbmc} was highly temperature-dependent and peaked at 60 °C (Figure 4C). Incubation at this temperature resulted in a 3-fold increase of TPA_{pbmc} compared to incubation at 37 °C. There was no significant TPA_{pbmc} observed at 70 °C.

3.2.2. Within-day and between-day precision

Average TPA_{pbmc} in samples spiked with 5 and 15 μg of PBMC cytosolic protein were 910 nmol/mg/h and 916 nmol/mg/h, respectively. The WDP and BDP of the TP assay were 8.1% and 4.6% in samples that were spiked with 5 μg protein. For samples spiked with 15 μg, the WDP and BDP were 9.2% and 6.0%.

3.2.3. Specificity

No significant interferences were observed. The deviations from the nominal thymine concentrations were between -0.3 and 6.5% (Supplementary table 1), showing that the specificity of this assay is acceptable.

3.2.4. Stability

Results of stability experiments are shown in Table 1. TPA_{pbmc} was stable in whole blood that was stored for 24 hours at room temperature. Storage of whole blood for 4 hours on ice-water resulted in significant reduction of
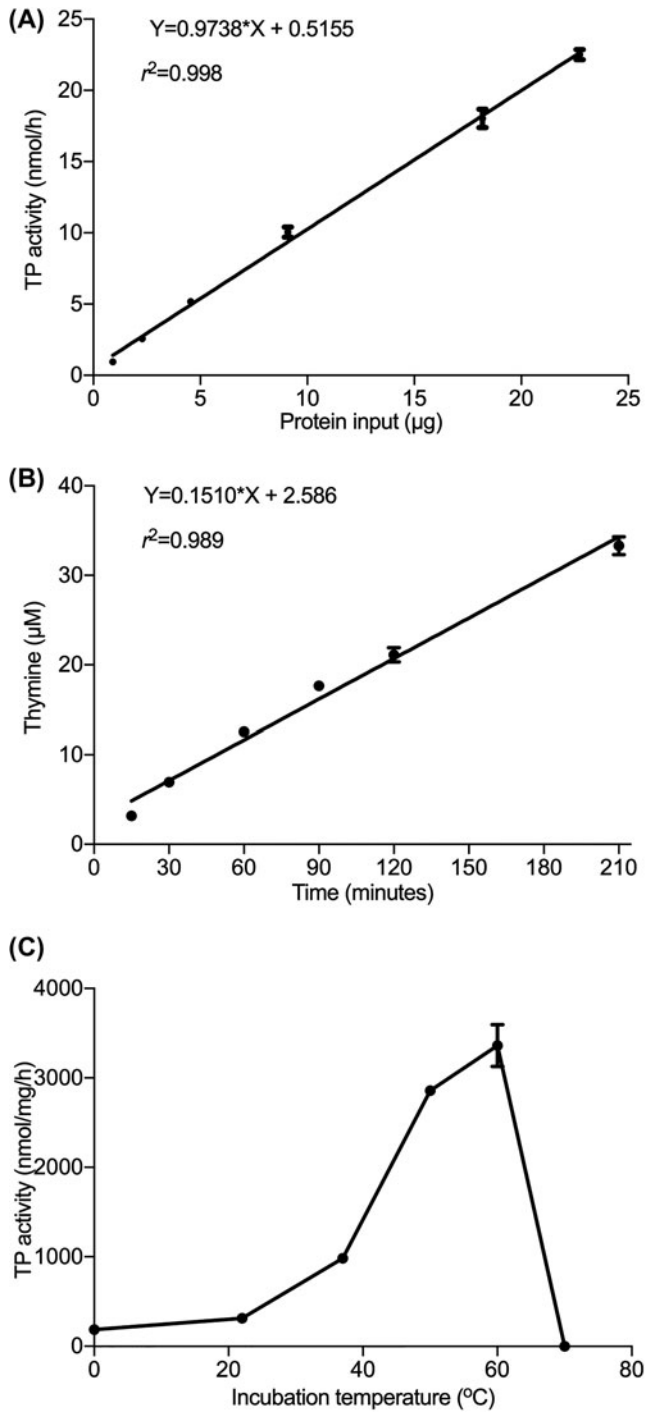
Figure 4. Linearity of thymidine phosphorylase activity in peripheral blood mononuclear cells with protein input (A) incubation time (B) and the effect of incubation temperature (C). Thymidine phosphorylase activity was determined in pooled samples from three volunteers. Results are expressed by the average $\pm$ s.d. of three samples. Abbreviation: TP, thymidine phosphorylase.

Table 1. Stability of thymidine phosphorylase activity in peripheral blood mononuclear cells at different processing stages and storage conditions.

Component	Condition	Initial TP activity (nmol/mg/h)	Measured TP activity (nmol/mg/h)	Deviation (%)	CV (%)
Whole blood	Ice-water, 4 hours	1020	792	−22.3	9.7
Whole blood	Ambient, 4 hours	1020	987	−3.2	5.9
Whole blood	Ambient, 24 hours	1020	1015	−0.5	2.0
PBMC dry pellet	−80 °C, 6 months	934	856	−8.4	5.5
PBMC cytosolic lysate	−80 °C, 6 months	950	871	−8.3	4.1
Re-injection stability (final extract)	Ambient, 24 hours	1020	1060	3.9	5.9
Assay buffer	−20 °C, 2 months	1129	1118	−1.0	3.8
Thymidine stock solution	−20 °C, 2 months	1041	1118	7.4	3.8

Abbreviations: TP, thymidine phosphorylase; CV, coefficient of variation; PBMC, peripheral blood mononuclear cell

TPA_{pbmc}. Long-term stability of PBMC dry pellets and cytosolic lysates was demonstrated for at least 6 months at -80 °C. There was no significant change in TPA_{pbmc} after storage of assay buffer and thymidine stock solutions for two months at -20 °C. Furthermore, re-injection analysis showed that final extracts were adequately stored in the HPLC autosampler for at least 24 hours at room temperature.

3.3. Clinical applicability

Figure 5 illustrates the TPA_{pbmc} of five patients. These analyses demonstrated the feasibility of quantification of TPA_{pbmc} in samples that were collected from cancer patients. TPA_{pbmc} demonstrated between-subject variability but did not seem to be altered after seven days of capecitabine treatment or at the end of treatment.
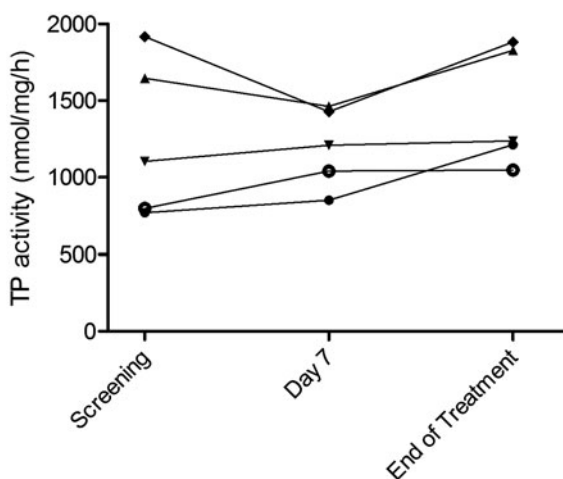

Figure 5. Thymidine phosphorylase activity in peripheral blood mononuclear cells of five cancer patients before treatment (Screening) with capecitabine, on the 7th day of capecitabine treatment and at the end of treatment. The patients participated in a phase I clinical trial of chronomodulated capecitabine therapy. Abbreviation: TP, thymidine phosphorylase.
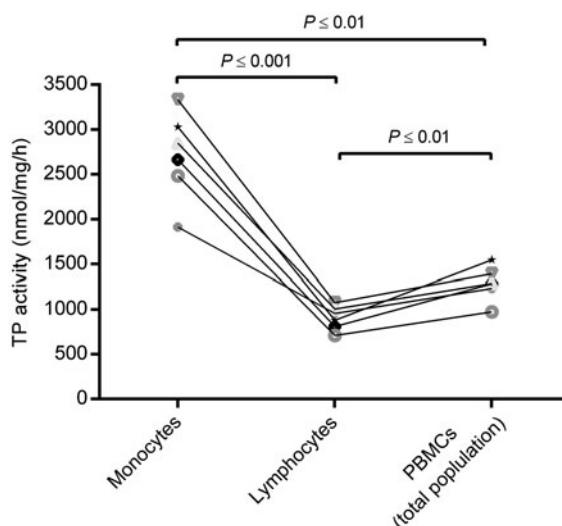
Figure 6 Thymidine phosphorylase activity in purified monocytes, purified lymphocytes and peripheral blood mononuclear cells of six volunteers. Abbreviation: TP, thymidine phosphorylase.

3.4. TP activity in monocyte and lymphocyte subpopulations

The TP activities in monocytes, lymphocytes and PBMCs of six volunteers are shown in Figure 6. Average (± s.d.) TP activity in monocytes was 2710 ± 490 nmol/mg/h and in lymphocytes 906 ± 134 nmol/mg/h ($P < 0.001$). The average TPA_{pbmc} was 1286 ± 190 nmol/mg/h and was significantly different from TP activity in lymphocytes and monocytes ($P < 0.01$). Purity of the monocyte and lymphocyte populations ranged between 93.5-98.6%. The percentage of monocytes in the total PBMC fractions ranged between 14.8-21.9%.

4. Discussion

The primary aim was to develop and validate a simple and accurate method for the quantification of TPA_{pbmc}. We successfully developed a method that is based on *ex vivo* conversion of the TP substrate thymidine to the reaction product thymine. By applying the widely available HPLC-UV technique, this method can be implemented in most laboratories. The use of the internal standard 5-BU helps to reduce variation due to sample preparation.

We found a small background signal at the retention time of thymine in the chromatogram of a sample of 2 mM thymidine. The thymine background signal was not noticeable in samples that did not contain the thymidine stock solution. Based on these results it can be concluded that thymine is an impurity in the thymidine dry powder, which is ≥99% pure, and directly causes the background signal. By quantifying the amount

of thymine in three negative control samples, thymine background signals can be easily quantified and corrected for in study samples.

Validation experiments showed that assay linearity was observed for PBMC cytosolic protein levels between 2.3 – 22.7 µg. Within this range, assay accuracy and precision met the predefined criteria and were considered acceptable. Using only 2.3 µg PBMC cytosolic protein, the amount of thymine formed highly exceeds background levels. The minimum amount of PBMC protein needed for quantification of TPA$_{pbmc}$ can be extracted from approximately 125,000 cells, which can already be isolated from about 0.1 mL of whole blood. Since only a limited amount of blood is required, the developed assay can be easily implemented for sequential quantification of TPA$_{pbmc}$ in cancer patients. Clinical applicability of the assay was confirmed by the quantification of TPA$_{pbmc}$ in five patients that were sampled at 3 time points during the treatment course.

The estimated K_m was 78.1 µM and is comparable to the previously determined K_m of TP activity in the total leukocyte population.[24] It can be anticipated that a thymidine concentration of 2 mM, which corresponds to approximately ~25 times the estimated K_m, saturation of the TP enzymes occurs. Therefore, a thymidine concentration of 2 mM can be considered adequate for quantification of TP in PBMCs. The V_{max} of TP activity is approximately 3-fold higher in PBMCs compared to TP activity in total leukocytes.[24]

Assay precision was tested using 5 and 15 µg PBMC protein. These protein input levels were at the lower and higher end, respectively, of the established linear range. For both protein input levels, the within-day and between-day precision met the predefined criteria for adequate precision. Therefore, we conclude that the assay shows good precision within the validated linear range of protein input levels.

Changes in temperature highly affect TPA$_{pbmc}$. Shaw et al. previously found that incubation at a temperature slightly above 40 °C increases the conversion of thymidine to thymine compared to samples that were incubated at 37 °C.[29] We also determined TPA$_{pbmc}$ at temperatures above 40 °C and found the highest TPA$_{pbmc}$ at 60 °C. In order to obtain reliable and reproducible results, it is of major importance to keep the incubation temperature constant. We choose to maintain the incubation temperature at 37 °C because this best resembles the *in vivo* physiological conditions, and may better predict the role of TP inducers or inhibitors on TP activity. In addition, future experiments might be performed to study the role of TP inducers or inhibitors on TP activity. Such experiments normally require incubation at a temperature that resembles the *in vivo* situation.

Previous studies demonstrated that changes in pH might affect TP enzyme kinetics.[24,29] Therefore, all experiments were performed using an

assay buffer with a fixed pH of 7.4. The pH of the freshly prepared assay buffer should be examined before usage.

Other assays have been described for quantification of TP activity of the total leukocyte population.[24,30] Leukocyte subpopulations, however, showed high variability in TP activity.[25] A more homogenous population, like PBMCs, is most probably more appropriate for longitudinal observation of TP activity than the total leukocyte population. The PBMC fraction consists for 70 – 90% of lymphocytes and for 10-30% of monocytes. We found that TP activity was ∼3 times higher in monocytes than in lymphocytes. Other researchers previously found that monocytes also possess higher DPD activity in comparison with lymphocytes.[31] Although the proportion of monocytes in PBMCs is relatively low, changes in the sample composition with respect to the relative amount of monocytes could affect the TP activity. Our analysis by flow cytometry provides a simple way for monitoring the amount of monocytes in the PBMC samples and their potential effect on TPA_{pbmc}.

A limitation of the method is that it is focused on the enzyme TP. The activities of other enzymes that are involved in the activation and degradation of capecitabine, such as DPD, TS and UP, are not determined using the current assay and warrant the use of other methods.

The applicability for quantification of TP might not be limited to prediction of fluoropyrimidine-induced toxicity. Fairbanks et al. previously described a quality control (QC) method for a pharmaceutical formulation of erythrocytes that were encapsulated with TP.[32] This QC method also employs the conversion of thymidine to thymine in order to quantify TP activity. The TP-loaded erythrocytes can be administered to patients with TP deficiency, a mutation that causes mitochondrial neurogastrointestinal encephalomyopathy.

Other researchers demonstrated the potential role of TP protein and gene expression as a predictor for patient prognosis[33,34] and sensitivity to capecitabine treatment.[35,36] TP expression is therefore a promising biomarker for the treatment of cancer patients. Additional clinical research is needed to determine the clinical validity and applicability of TP phenotyping methods.

In conclusion, we successfully developed and validated a simple, accurate and precise assay for quantification of TPA_{pbmc}. Clinical applicability of the assay was demonstrated. Therefore, we believe that the developed assay is suitable for quantification of TPA_{pbmc} in clinical studies.

Conflicts of interest

The authors declare that they have no conflict of interest.

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References

[1]   Mackean M, Planting A, Twelves C, et al. Phase I and pharmacologic study of intermittent twice-daily oral therapy with capecitabine in patients with advanced and/or metastatic cancer. *J Clin Oncol*. **1998**,, *16*:2977–2985.

[2]   Judson IR, Beale PJ, Trigo JM, et al. A human capecitabine excretion balance and pharmacokinetic study after administration of a single oral dose of 14C-labelled drug. *Invest New Drugs*. **1999**,, *17*:49–56.

[3]   Budman DR, Meropol NJ, Reigner B, et al. Preliminary studies of a novel oral fluoropyrimidine carbamate: capecitabine. *J Clin Oncol*. **1998**, *16*:1795–802.

[4]   Miwa M, Ura M, Nishida M, et al. Design of a novel oral fluoropyrimidine carbamate, capecitabine, which generates 5 fluorouracil selectively in tumours by enzymes concentrated in human liver and cancer tissue. *Eur J Cancer*. **1998**, *34*:1274–1281. doi: 10.1016/S0959-8049(98)00058-6

[5]   Tabata T, Katoh M, Tokudome S, et al. Bioactivation of capecitabine in human liver: involvement of the cytosolic enzyme on 5'-deoxy-5-fluorocytidine formation. *Drug Metab Dispos*. **2004**, *32*:762–7.

[6]   Walko CM, Lindley C   Capecitabine: a review. *Clin Ther*. **2005**, *27*:23–44. doi: 10.1016/j.clinthera.2005.01.005

[7]   Wilson PM, Danenberg P V., Johnston PG, et al. Standing the test of time: targeting thymidylate biosynthesis in cancer therapy. *Nat Rev Clin Oncol*. **2014**, *11*:282–98. doi: 10.1038/nrclinonc.2014.51

[8]   Ishikawa T, Sekiguchi F, Fukase Y, et al. Positive correlation between the efficacy of capecitabine and doxifluridine and the ratio of thymidine phosphorylase to dihydropyrimidine dehydrogenase activities in tumors in human cancer xenografts. *Cancer Res*. **1998**, *58*:685–90.

[9]   Schüller J, Cassidy J, Dumont E, et al. Preferential activation of capecitabine in tumor following oral administration to colorectal cancer patients. *Cancer Chemother Pharmacol*. **2000**, *45*:291–7.

[10]   Longley DB, Harkin DP, Johnston PG   5-fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer*. **2003**, *3*:330–8. doi: 10.1038/nrc1074

[11]   Midgley R, Kerr DJ   Capecitabine: have we got the dose right? *Nat Clin Pract Oncol*. **2009**, *6*:17–24. doi: 10.1038/ncponc1240

[12]   Deenen MJ, Meulendijks D, Cats A, et al. Upfront Genotyping of DPYD*2A to Individualize Fluoropyrimidine Therapy: A Safety and Cost Analysis. *J Clin Oncol*. **2016**, *34*:227–34. doi: 10.1200/JCO.2015.63.1325

[13]   Meulendijks D, Henricks LM, Sonke GS, et al. Clinical relevance of DPYD variants c.1679T > G, c.1236G > A/HapB3, and c.1601G > A as predictors of severe fluoropyrimidine-associated toxicity: a systematic review and meta-analysis of individual patient data. *Lancet Oncol*. **2015**, *16*:1639–50. doi: 10.1016/S1470-2045(15)00286-7

[14]   Jennings BA, Loke YK, Skinner J, et al. Evaluating predictive pharmacogenetic signatures of adverse events in colorectal cancer patients treated with fluoropyrimidines. *PLoS One*. **2013**, *8*:e78053. doi: 10.1371/journal.pone.0078053

[15]   Milano G, Etienne-Grimaldi M-C, Mari M, et al. Candidate mechanisms for capecitabine-related hand-foot syndrome. *Br J Clin Pharmacol*. **2008**, *66*:88–95. doi: 10.1111/j.1365-2125.2008.03159.x

[16] Evrard A, Cuq P, Ciccolini J, et al. Increased cytotoxicity and bystander effect of 5-fluorouracil and 5-deoxy-5-fluorouridine in human colorectal cancer cells trans-fected with thymidine phosphorylase. *Br J Cancer.* **1999**, *80*:1726–33. doi: 10.1038/sj.bjc.6690589

[17] Toi M, Bando H, Horiguchi S, et al. Modulation of thymidine phosphorylase by neoadjuvant chemotherapy in primary breast cancer. *Br J Cancer.* **2004**, *90*:2338–2343. doi: 10.1038/sj.bjc.6601845

[18] Han J-Y, Hong EK, Lee SY, et al. Thymidine phosphorylase expression in tumour cells and tumour response to capecitabine plus docetaxel chemotherapy in non-small cell lung cancer. *J Clin Pathol.* **2005**, *58*:650–4. doi: 10.1136/jcp.2004.022764

[19] Puglisi F, Andreetta C, Valent F, et al. Anthracyclines and taxanes induce the upregulation of thymidine phosphorylase in breast cancer cells. *Anticancer Drugs.* **2007**, *18*:883–8. doi: 10.1097/CAD.0b013e32816ebede

[20] Zhang J, Gu SY, Gan Y, et al. Vinorelbine and capecitabine in anthracycline- and/or taxane-pretreated metastatic breast cancer: Sequential or combinational? *Cancer Chemother Pharmacol.* **2013**, *71*:103–113. doi: 10.1007/s00280-012-1983-y

[21] Sawada N, Ishikawa T, Sekiguchi F, et al. X-ray irradiation induces thymidine phosphorylase and enhances the efficacy of capecitabine (Xeloda) in human cancer xenografts. *Clin Cancer Res.* **1999**, *5*:2948–53.

[22] Zhang S-H, Zhang H, He H-W, et al. Lidamycin up-regulates the expression of thymidine phosphorylase and enhances the effects of capecitabine on the growth and pulmonary metastases of murine breast carcinoma. *Cancer Chemother Pharmacol.* **2013**, *72*:777–88. doi: 10.1007/s00280-013-2253-3

[23] Di Gennaro E, Piro G, Chianese MI, et al. Vorinostat synergises with capecitabine through upregulation of thymidine phosphorylase. *Br J Cancer.* **2010**, *103*:1680–91. doi: 10.1038/sj.bjc.6605969

[24] van Kuilenburg ABP, Zoetekouw L   Determination of thymidine phosphorylase activity by a non-radiochemical assay using reversed-phase high-performance liquid chromatography. *J Chromatogr B.* **2005**, Analyt Technol Biomed Life Sci *820*:271–275. doi: 10.1016/j.jchromb.2005.04.009

[25] van Kuilenburg ABP, Zoetekouw L   Determination of thymidine phosphorylase activity in human blood cells and fibroblasts by a nonradiochemical assay using reversed-phase high-performance liquid chromatography. *Nucleosides Nucleotides Nucleic Acids.* **2006**, *25*:1261–4. doi: 10.1080/15257770600889006

[26] Pluim D, Schilders KAA, Jacobs BAW, et al. Pharmacodynamic assay of thymidylate synthase activity in peripheral blood mononuclear cells. *Anal Bioanal Chem.* **2013**, *405*:2495–2503. doi: 10.1007/s00216-012-6676-1

[27] Pluim D, Jacobs BAW, Krähenbühl MD, et al. Correction of peripheral blood mononuclear cell cytosolic protein for hemoglobin contamination. *Anal Bioanal Chem.* **2013**, *405*:2391–2395.

[28] Pluim D, Jacobs BAW, Deenen MJ, et al. Improved pharmacodynamic assay for dihydropyrimidine dehydrogenase activity in peripheral blood mononuclear cells. *Bioanalysis.* **2015**, *7*:519–29. doi: 10.4155/bio.14.304

[29] Shaw T, Smillie RH, MacPhee DG   The role of blood platelets in nucleoside metabolism: assay, cellular location and significance of thymidine phosphorylase in human blood. *Mutat Res.* **1988**, *200*:99–116.

[30] Martí R, López LC, Hirano M   Assessment of thymidine phosphorylase function: measurement of plasma thymidine (and deoxyuridine) and thymidine phosphorylase activity. *Methods Mol Biol.* **2012**, *837*:121–33. doi: 10.1007/978-1-61779-504-6_8

[31] Van Kuilenburg AB, Van Lenthe H, Tromp A, et al. Pitfalls in the diagnosis of patients with a partial dihydropyrimidine dehydrogenase deficiency. *Clin Chem.* **2000** *46*:9–17.

[32] Fairbanks LD, Levene M, Bax BE Validation of a HPLC method for the measurement of erythrocyte encapsulated thymidine phosphorylase (EE-TP) activity. *J Pharm Biomed Anal.* **2013**, *76*:8–12. doi: 10.1016/j.jpba.2012.12.006

[33] Minisini AM, Pascoletti G, Intersimone D, et al. Expression of thymidine phosphorylase and cyclooxygenase-2 in melanoma. *Melanoma Res.* **2013**, *23*:96–101. doi: 10.1097/CMR.0b013e32835e7734

[34] Bonotto M, Bozza C, Di Loreto C, et al. Making capecitabine targeted therapy for breast cancer: which is the role of thymidine phosphorylase? *Clin Breast Cancer.* **2013**, *13*:167–72. doi: 10.1016/j.clbc.2012.10.002

[35] Andreetta C, Puppin C, Minisini A, et al. Thymidine phosphorylase expression and benefit from capecitabine in patients with advanced breast cancer. *Ann Oncol.* **2009**, *20*:265–71. doi: 10.1093/annonc/mdn592

[36] Puglisi F, Cardellino GG, Crivellari D, et al. Thymidine phosphorylase expression is associated with time to progression in patients receiving low-dose, docetaxel-modulated capecitabine for metastatic breast cancer. *Ann Oncol.* **2008**, *19*:1541–6. doi: 10.1093/annonc/mdn165