Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 816 (2005) 183–192
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

High-performance liquid chromatographic assay for the determination of total and free topotecan in the presence and absence of anti-topotecan antibodies in mouse plasma

Jin Chen, Joseph P. Balthasar*

Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, University at Buffalo, Buffalo, NY 14260, USA

Received 13 April 2004; accepted 16 November 2004
Available online 8 December 2004

Abstract

A rapid and sensitive high-performance liquid chromatographic (HPLC) assay has been developed to allow determination of total (i.e. bound and unbound) and free (i.e. unbound) topotecan (TPT) in mouse plasma in the presence and absence of anti-TPT antibodies. The chromatographic analysis was carried out using reversed-phase isocratic elution with a Nova-Pak C18 column (3.9 mm × 150 mm, 4 μm) protected by a Nova-Pak C18 guard column (3.9 mm × 20 mm, 4 μm), where 10 mM KH_2PO_4–methanol–triethylamine (72:26:2 (v/v/v), pH 3.5) was used as the mobile phase. Topotecan was quantified with fluorescence detection using an excitation wavelength of 361 nm and an emission wavelength of 527 nm. The retention time for the internal standard, acridine, and TPT were 7.4 and 9.0 min, respectively. The lower limit of quantitation (LOQ) for TPT was determined as 0.02 ng in mouse plasma and mouse plasma ultrafiltrate, corresponding to a concentration of 1 ng/ml in 20 μl mouse plasma. The assay was shown to be linear over a concentration range of 1–500 ng/ml. The recoveries of free and total TPT from spiked mouse plasma were within 10% of theoretical values (assessed at 1, 20 and 500 ng/ml). The validated HPLC assay was applied to evaluate TPT pharmacokinetics following administration of TPT to Swiss Webster mice and to hyperimmunized and control BALB/c mice. The assay has been shown to be capable for measuring total and free TPT in mouse plasma with high sensitivity and will allow the testing of the effect of anti-TPT antibodies on the disposition of TPT.

Keywords: Topotecan; Anti-TPT antibodies; HPLC

1. Introduction

Ovarian cancer is the most prominent form of gynecologic cancer, with approximately 20,000 new cases reported each year in the United States. The majority of ovarian cancer patients are diagnosed with advanced, disseminated, intra-peritoneal (i.p.) disease. Standard chemotherapy for advanced ovarian cancer results in median progression-free survival of 16–22 months and 5-year overall survival of 20–30% [1–3]. Because ovarian cancer usually remains confined in the peritoneal cavity, intra-peritoneal chemotherapy has been investigated as an approach to increase the efficacy and decrease the toxicity of ovarian cancer chemotherapy [4]. Unfortunately, following i.p. chemotherapy, drug is typically absorbed into the systemic circulation rapidly, and drug-induced systemic toxicities prevent administration of sufficient doses of chemotherapy to eradicate the peritoneal disease.

This laboratory is investigating a drug targeting strategy that employs anti-drug antibodies (ADAb) to minimize systemic drug exposure and reduce the severity of systemic drug toxicity following i.p. chemotherapy [5,6]. The approach utilizes i.p. administration of chemotherapeutic drug with simultaneous i.v. administration of anti-drug antibodies. Drug that diffuses out of the peritoneum and enters into the systemic circulation may be rapidly bound by ADAb due to the

* Corresponding author. Tel.: +1 716 645 2842x256; fax: +1 716 645 3693.
E-mail address: jb@acsu.buffalo.edu (J.P. Balthasar).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.11.035

high specificity and affinity of the ADAb to the drug, leading to dramatic decreases in free drug concentrations in the systemic circulation. As such, this "inverse targeting strategy" attempts to increase the selectivity of drug therapy by decreasing the efficiency of drug delivery to sites associated with drug toxicities (i.e. in contrast with "traditional targeting strategies", which attempt to increase the selectivity of drug therapy by increasing the efficiency of drug delivery to sites associated with desired effects).

The feasibility of this approach has been examined in rodents using methotrexate as a model drug. Systemic administration of anti-methotrexate Fab fragments (AMF) was found to enhance pharmacokinetic selectivity of i.p. methotrexate therapy, increasing the ratio of peritoneal to systemic exposure to unbound drug [6]. With this combination therapy, AMF was found to be able to reduce MTX toxicity [7], allow a greater than five-fold increase in the maximum tolerated dose of MTX, and enhance the median survival time of mice bearing peritoneal tumors (Lobo and Balthasar, in preparation).

Having established the feasibility of the approach, we are now interested in applying this targeting strategy to enhance the selectivity of topotecan (TPT) chemotherapy. Topotecan (*s*-9-dimethylaminomethyl-10-hyderoxycampthothecin) (Fig. 1) has been selected due to its high potency, and due to its status as a preferred therapy for refractory, metastatic ovarian cancer [8–11]. Additionally, two Phase I studies have demonstrated the feasibility of i.p. TPT chemotherapy in patients with advanced peritoneal cancer [12,13].

To facilitate testing hypotheses related to the optimization of i.p. topotecan chemotherapy in murine models of ovarian cancer, it was necessary to develop a sensitive assay capable of accurately quantifying both total and free topotecan in mouse plasma. Previously published HPLC methods have described the measurement of TPT in human plasma [14–16], rat plasma [17], and dog plasma [17]. These validated HPLC assays require relatively large volumes of plasma per sample (i.e. 100–250 μl human plasma; 100 μl rat or dog plasma), which would prevent their application to mouse studies involving serial sampling. Additionally, there are no reports of validated HPLC assays for quantitation of total and free TPT concentrations in the presence of anti-TPT antibodies.

In this paper, we describe the development and validation of a rapid and sensitive HPLC assay for the determination of the TPT concentrations in mouse plasma in the presence and absence of anti-topotecan antibodies. The assay was used to characterize the pharmacokinetics of topotecan following topotecan administration to mice, including mice hyperimmunized to produce anti-topotecan antibodies.

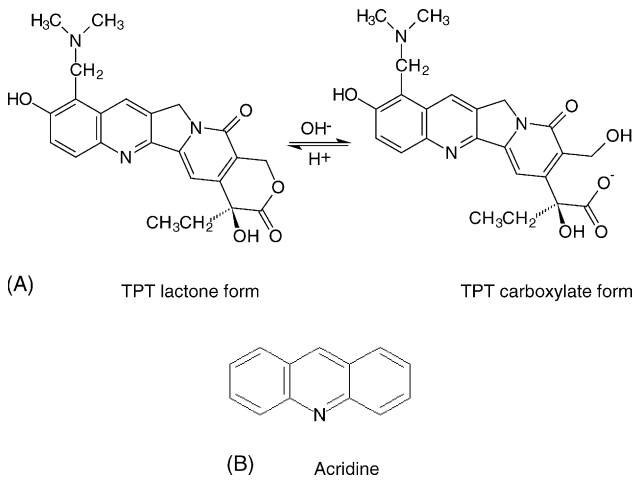

Fig. 1. (A) The pH-dependent hydrolysis equilibrium between TPT lactone and carboxylate forms. (B) The chemical structure of acridine.

2. Experimental

2.1. Chemicals and reagents

TPT, as the hydrochloride salt (SK&F S-104864-A), was provided by Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, National Institutes of Health (Bethesda, MD, USA). Triethylamine, acridine, potassium dihydrogenphosphate, phosphoric acid and methanol were purchased from Sigma (St. Louis, MO, USA). Imject mariculture keyhole limpet hemacyanin (mcKLH) was obtained from Pierce (Rockford, IL, USA). All other reagents were analytical grade.

2.2. HPLC instrumentation and conditions

The HPLC system (Waters, Milford, MA, USA) consisted of a Model 510 pump, a 717 plus autosampler, and a 474 fluorescence detector. Reversed-phased chromatography was performed with a Nova-Pak C18 column (3.9 mm × 150 mm, 4 μm) protected by a Nova-Pak C18 guard column (3.9 mm × 20 mm, 4 μm), and using an isocratic mobile phase consisting of 10 mM KH_2PO_4 water–methanol–triethylamine (72:26:2 (v/v/v), pH adjusted to 3.5 with phosphoric acid). The mobile phase was filtered (0.45 μm) and degassed prior to use. The flow rate was 1.0 ml/min and the injection volume was 10 μl. Fluorescence detection was set for an excitation wavelength of 361 nm and an emission wavelength of 527 nm with a 40 nm bandwidth; the digital filter was set at 10 s. Waters Millennium 3.2 software was used for instrument control, data acquisition, and data processing.

2.3. Sample preparation for calibration standards and QCs

Stock solutions of TPT, 100 μg/ml, were prepared in methanol and stored at −80 °C. Stock solutions of 50 ng/ml

of the internal standard, acridine, were prepared in methanol and stored at −20 °C. Working solutions of TPT containing 1, 2.5, 5, 10, 25 μg/ml, 25, 50, 100, 250 and 500 ng/ml were prepared by serial dilution of the internal stock solution with methanol. Calibration samples were prepared by combining 20 μl blank mouse plasma with 2 μl TPT working solution and 2 μl internal standard (50 ng/ml acridine). Samples were de-proteinized by the addition of 56 μl ice-cold methanol and by acidification with 20 μl 100 mM H_3PO_4. TPT undergoes reversible conversion between a lactone form and a carboxylate form (Fig. 1) [14]. In addition to allowing removal of proteins, acidification allows conversion of TPT carboxylate to TPT lactone. As such, the assay is quantifying TPT as the sum of the carboxylate and lactone forms. Calibration curves were prepared using standards of 1, 2, 5, 10, 20, 50, 100, 200 and 500 ng/ml. Samples were vortexed for 10 s, centrifuged at 10,000 × *g* for 10 min at 4 °C, and the resulting supernatant was injected onto the HPLC column. Quality control samples (QCs) for TPT at 1 ng/ml, 20 ng/ml, and 500 ng/ml using Swiss Webster mouse plasma and BALB/c mouse plasma were prepared via the same procedure.

For determination of TPT in the presence of anti-TPT antibodies, assay were validated by preparing QCs at 1, 20, and 500 ng/ml, using 20 μl of hyperimmunized BALB/c mouse sera. For determination of free TPT in mouse plasma, 20 μl of Swiss Webster mouse plasma ultrafiltrate was used. Mouse plasma ultrafiltrate was obtained by ultrafiltration in centrifree tubes (10 kD molecular weight cutoff, Bellerica, MA, USA), following centrifugation at 1500 × g for 10 min at 25 °C. The subsequent procedure was the same as described for total TPT in mouse plasma.

2.4. Assessment of non-specific adsorption of TPT to centrifree membrane

To assess the possible non-specific adsorption of TPT to the centrifree membrane, a standard curve in 25 mM KH_2PO_4 buffer, pH 3.0, was prepared. QC samples of TPT in buffer ultrafiltrate were made by ultrafiltration after spiking TPT working solution into buffer solution. The resultant ultrafiltrate was processed as described above.

2.5. Assay validation

2.5.1. Linearity of the calibration curve

The linearity of calibration curve was evaluated by linear regression of peak area ratios (TPT/acridine) versus TPT concentrations in mouse plasma samples. The assay was judged to be linear if the correlation coefficient *r* was greater than 0.995 by the least-square method.

2.5.2. Lower limit of quantitation

The lower limit of quantification was defined as the QC concentration associated with a mean assayed concentration that was within 10% of the nominal value and where the coefficient of variation was less than 15% for replicates of the QC, during both within- and between-day validation.

2.5.3. Precision, accuracy and recovery

The assay was assessed by intra- and inter-day accuracy and precision quantifying TPT at 1, 20, and 500 ng/ml concentrations. Accuracy was determined by comparing the calculated concentration using calibration curves to nominal concentrations. Intra-day variability was assessed through the analysis of QCs in triplicates, and inter-day variability was determined through the analysis of QCs on three different days.

2.6. Stability

TPT samples were prepared in mouse plasma and in acidified methanol extracts of plasma and were then stored at room temperature (∼20 °C), 4, −20 and −80 °C. Aliquots were assayed for TPT at selected time points up to 24 h. TPT was considered to be stable under the storage condition if the assayed percent recovery was found to be 85–115% of the nominal initial concentration.

2.7. Pharmacokinetics of TPT in mice

TPT pharmacokinetics were assessed following i.p. bolus injection of TPT at a dose of 10 mg/kg to a group of three male Swiss Webster mice. Blood samples (20–40 μl) were obtained through the saphenous vein at 5, 10, 30 min, 1, 2, 4, 12, and 24 h. Samples were transferred to heparinized microcentrifuge tubes and immediately centrifuged at 10,000 × *g* for 2 min. Plasma samples were stored in −80 °C and analyzed within 1–2 days. Plasma samples were diluted with blank Swiss Webster mouse plasma, if needed, and analyzed. Non-compartmental analysis was conducted via WinNonLin 2.1 software (Pharmsight, CA, USA).

2.8. Pharmacokinetics of TPT in the absence and presence of anti-TPT antibodies in mice

2.8.1. Preparation of immunogen and immunization

TPT was conjugated to mariculture keyhole limpet hemacyanin via the Mannich reaction using Pierce PharmLink Immunogen Kit (Pierce, Rockford, IL). Briefly, 0.9 mg TPT was linked with 2 mg imject mariculture keyhole limpet hemacyanin (KLH) by condensation with formaldehyde. The reaction was incubated at 50 °C over night, followed by dialysis against PBS for 2 h at room temperature (twice) and at 4 °C over night. The conjugate, KLH-TPT, was diluted with PBS and stored at −20 °C. The degree of conjugation was estimated by UV spectroscopy at 411 nm for topotecan and 280 nm for KLH. The conjugation ratio was estimated to be 70–100 topotecan molecules per KLH molecule.

Fifty micrograms KLH-TPT was dissolved in PBS and emulsified with an equal volume of Freund's incomplete

adjuvant (Sigma, St. Louis, MO, USA). Female BALB/c mice (Harlan, Indianapolis, IN) were immunized with 200 μl emulsion by i.p. injection every 3 weeks. The animals were bled via the saphenous vein 7 days after each dose of KLH-TPT. Whole blood was collected, heparinized and centrifuged. The resulting plasma were stored at −20 °C.

2.8.2. Screening of antiserum for anti-TPT antibodies activities

The formation of anti-TPT antibodies by the immunized mice was assessed by a traditional titer method detected by HPLC. The anti-TPT titer was defined as the dilution of the plasma at which 60 μl of diluted plasma produced a 50% of reduction of the TPT peak area. Plasma samples were prepared by dilution with normal mouse plasma spiked with TPT to a final concentration of 200 ng/ml. Samples were incubated at room temperature for 1 h and then ultrafiltrated at 1500 g for 10 min. The resulting ultrafiltrate was processed following the procedure described before.

2.8.3. Verification of antibody binding in hyperimmunized BALB/c plasma via protein-G adsorption

To verify the presence of anti-TPT antibody in hyperimmunized BALB/c plasma, TPT concentrations were assessed from plasma samples before and after treatment with a protein-G affinity column (HiTrap Protein-G, Pharmacia Biotech Inc., Piscataway, NJ). Briefly, BALB/c mouse plasma was collected from hyperimmunized and non-immunized mice. The plasma samples were diluted 2×, 5× and 20× with PBS, 'spiked' with TPT, and then incubated at room temperature for 2 h. Aliquots of each sample, 0.9 ml, were loaded onto the protein-G column. The column was then washed with 1.8 ml of 20 mM Na_2HPO_4, pH 7.0, and the column eluate (2.7 ml) was collected. As such, each sample was diluted by an additional factor of 3×, leading to final dilution factors of 6×, 15× and 60×, respectively. Due to the high affinity of protein-G for immune gamma globulin (IgG), this treatment was expected to separate IgG antibody-bound TPT (i.e. which would be retained on the column) from TPT that was either unbound or bound to other plasma proteins (i.e. which would elute from the column during washing). TPT concentrations in plasma aliquots and in samples of the column eluate were determined through the use of the HPLC, and the fraction of TPT not bound to IgG antibodies was calculated as the ratio between concentration of TPT after protein-G treatment and the concentration determined before protein-G treatment. Additionally, TPT-spiked hyperimmunized plasma samples were diluted 6×, 15× and 60× with PBS, and ultrafiltrated. TPT concentrations in the ultrafiltrate were determined (as described above), and the fraction of unbound TPT was calculated as the ratio between concentration of TPT in the ultrafiltrate over that before ultrafiltration treatment. SDS–PAGE was performed to test the presence of IgG antibodies in plasma, in column eluate, and in plasma ultrafiltrate.

2.8.4. Pharmacokinetics of TPT in the presence and absence of anti-TPT antibodies

TPT pharmacokinetics were evaluated following i.v. bolus injection of TPT at a dose of 10 mg/kg to two groups of BALB/c mice. One group was immunized with KLH-TPT and the other group consisted of non-immunized, control BALB/c mice. Blood samples (20–40 μl) were obtained through the saphenous vein at 10, 30 min, and at 1, 2, 4 h. Animals were sacrificed at 8 h, and blood (300–700 μl) was collected by cardiac puncture. Blood samples were collected in heparinized microcentrifuge tubes and immediately centrifuged at 10,000 × *g* for 2 min. Plasma samples were analyzed for total TPT concentration. The 8 h samples were analyzed for both total and free TPT concentrations. Noncompartmental analysis was conducted via WinNonLin 2.1 software.

3. Results and discussion

3.1. Development of the assay

TPT is a semi-synthetic, water-soluble analog of the plant alkaloid camptothecin (CPT). It undergoes a pH-dependent reversible hydrolysis, yielding a hydroxyl carboxylate form (Fig. 1). Under acidic conditions (pH < 4), the lactone predominates. When pH is greater than 10, the ring is quantitatively opened. At physiological pH (pH 7.4), the concentration of the carboxylate form exceeds the concentration of the lactone form by a factor of approximately 2:1. However, it is believed that the lactone form is the biologically active form [18]. In clinical studies, it has been reported that over a wide dose range of 2.5–22.5 mg/m^2, the AUC ratio between TPT lactone and total TPT (i.e. the sum of the lactone and carboxylate) remained relatively constant (∼0.33) [19]. In spite of the evidence suggesting that the lactone is the active form of TPT, several studies have shown that concentrations of total TPT, and not TPT lactone, correlates better with TPT-induced effects [20,21]. This finding is likely related to the poor stability of TPT lactone in plasma and in methanol extracts. In unacidified methanol extracts, the TPT lactone is associated with a decay half-life of 200 min at room temperature. On the other hand, TPT has been found to be stable in acidified methanol extracts for over 4.5 month at −30 °C and stable at room temperature for 24 h [20]. Consequently, we structured the present assay to measure TPT concentrations as the sum of the lactone and the carboxylate forms.

Following administration of TPT to man, the majority of TPT is eliminated unchanged in urine [9], and metabolism represents a minor pathway for TPT elimination. Three TPT metabolites have been identified: *N*-desmethyl topotecan [22], TPT-*O*-glucuronide and *N*-desmethyl TPT-*O*-glucuronide [23]. These TPT metabolites are not commer-

cially available and, consequently, we were not able to assess the assay selectivity in the presence of the TPT metabolites. However, only low concentrations of these metabolites have been found in blood. For example, following i.v. infusion for 30 min of 1.5 mg/m^2 TPT on a daily × 5/3 week schedule, only 2.0, 1.9, and 0.7% of the TPT dose was eliminated in the urine as *N*-desmethyl TPT, TPT-*O*-glucuronide, and *N*-desmethyl TPT-*O*-glucuronide, respectively [23]. Additionally, Rosing et al. have shown that when TPT was given by 30 min i.v. infusion at 1.0 mg/m^2, maximal plasma concentrations of *N*-desmethyl topotecan were only 0.7% of the maximal plasma concentrations of TPT [22]. As such, although we have not assessed assay response to TPT metabolites, it is not likely that the metabolites will be present in sufficient concentrations to affect the accuracy or precision of our assay.

Validated HPLC assays for determination of TPT in human plasma have appeared in several publications. The first and most frequently cited assay was published in 1990 for determination of TPT lactone and carboxylate forms [14]. However, the assay was complicated with an "endogenous peak" that overlaps with the peak associated with the TPT carboxylate, and subtraction of the interfering peak was necessary for determination of the concentrations of carboxylate. In 1995, an improved method for measurement of the total TPT in human plasma was developed, which called for preparation of an acidified methanol extract of plasma with 7% $HClO_4$ [17]. However, interfering substances prevented application of this assay to rat and dog pharmacokinetic studies. To assay the plasma concentrations of TPT in these animals, a reduced column temperature (19 °C) was needed. Among all the validated assays, the assay of Rosing and co-workers was associated with the highest sensitivity, with a limit of quantitation of 0.005 ng (on column) [24]. In the present work, we have modified the assay of Rosing et al. for application in our mouse studies.

The Rosing et al. report utilized a mobile phase comprised of methanol:hexane-1-sulfonic acid: *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED). In the present assay, we have used methanol:KH_2PO_4:TEA, as we found that, in our hands, this mobile phase allowed superior sensitivity, with a desirable capacity factor (Fig. 2).

In reviewing previous assays for quantitation of TPT in human/animal plasma, we found that published assays employed a wide range of different mobile phase pH levels (e.g.

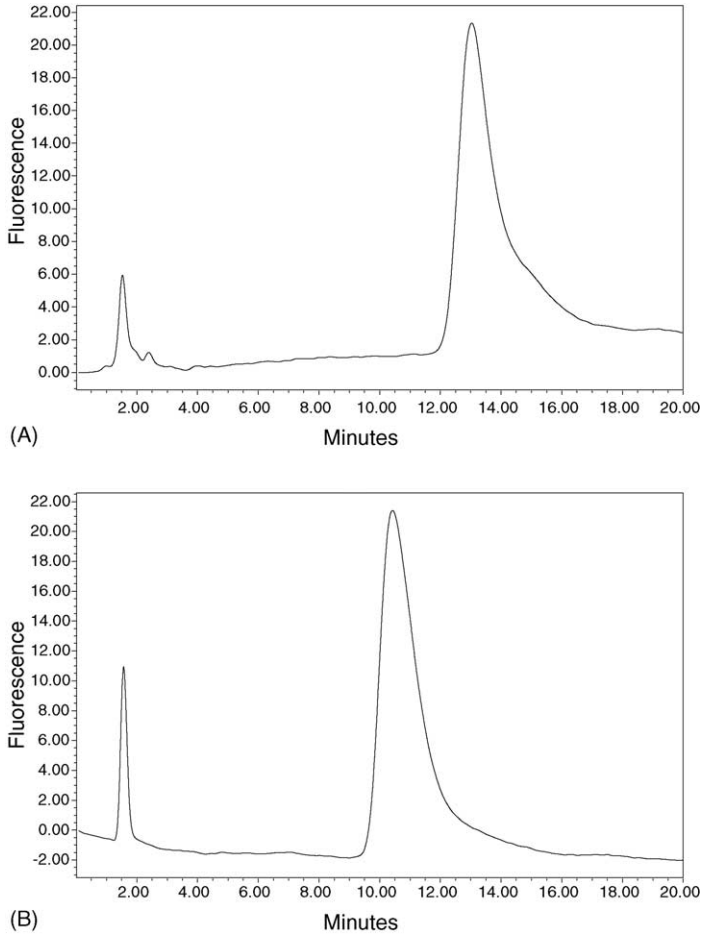

Fig. 2. HPLC profiles for the comparison of mobile phase with 10 ng/ml TPT spiked in methanol. (A) Mobile phase, MeOH:0.1 M hexane-1-sulfonic acid in MeOH:0.01 M TEMED = 25:10:65 (v/v/v), pH 6.0; (B) mobile phase, 0.01 M KH_2PO_4:MeOH:TEA = 72:27:1 (v/v/v), pH 6.0.

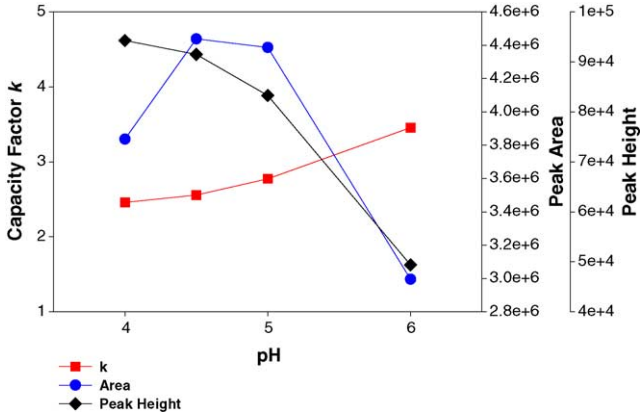

Fig. 3. The effect of mobile phase pH on capacity factor, peak area and peak height with 10 ng/ml TPT spiked in methanol. With a fixed concentration of TEA (1%), as the pH increased, the capacity factor *k* also increased slightly.

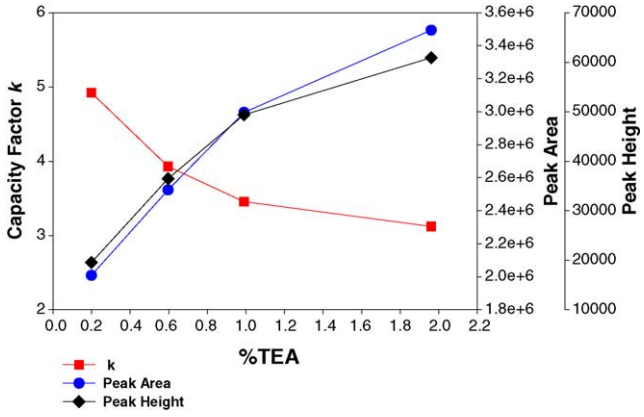

Fig. 4. The effect of triethylamine (TEA) percentage in the mobile phase on capacity factor, peak area and peak height with 10 ng/ml TPT spiked in methanol. At pH of 6.0, as the percentage of TEA was increased, an improved sharp TPT peak associated with a decreased capacity factor *k* was observed.

pH 3.0 [16]; pH 6.0 [15,17,24]; pH 6.5 [25]). In order to find an optimal pH for use in our system, preliminary studies were conducted to evaluate the effects of pH (from 4.0 to 6.0) and the effect of the percentage of TEA (from 0.2 to 2%) on the capacity factor *k*, TPT peak area, and TPT peak height. We observed that, with a fixed concentration of TEA (1%), as the pH increased, the capacity factor *k* also increased slightly (Fig. 3). At pH of 6.0, as the percentage of TEA was increased, an improved sharp TPT peak associated with a decreased capacity factor *k* was observed (Fig. 4). This is likely due to the blockage of ionized silanols in the Nova-Pak C18 column by TEA. Based on these preliminary results, we adjusted the pH of the mobile phase to 3.5 and used TEA at a concentration of 2%. This mobile phase allowed adequate peak shapes and retention times for both TPT and acridine (Fig. 5). Further modifications to the assay procedure to reduce TPT retention time resulted in a loss of assay resolution (data not shown).

In previous studies of human, rat, and dog plasma, internal standards were rarely used, presumably due to the difficulties associated with the identification of suitable compounds. In our effort to find an internal standard for TPT, we initially tried camptothecin and CPT analogues, irinotecan (CPT-11) and SN 38. However, consistent with a previous report [16], these agents were associated with either poor stability and/or undesirable capacity factors (data not shown). However, Subramanian et al. reported that acridine served as a suitable internal standard within an assay employing a complex of four-element mo-

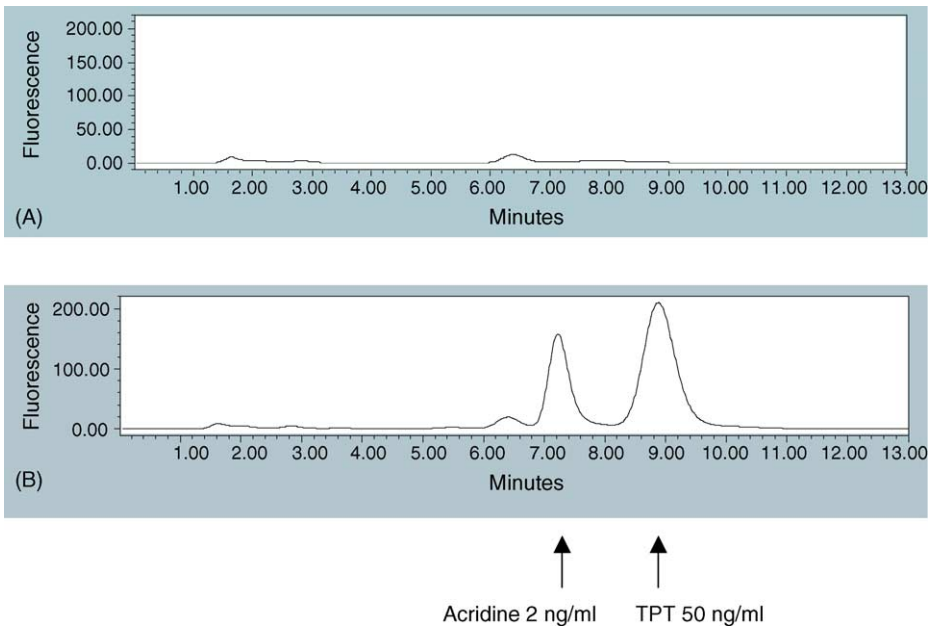

Fig. 5. Typical chromatograms obtained from HPLC analysis of mouse plasma. (A) The blank mouse plasma shows apparent clear background after 6.8 min. (B) The spiked acridine and TPT are well separated with retention time (t_R) of acridine (7.4 min) and TPT (9.0 min).

Table 1
The accuracy and precision (CV%) associated with assayed concentrations of quality control samples for total TPT in the absence of anti-TPT antibodies

QC concentration (ng/ml)	Assayed concentration (ng/ml)	Recovery (%)	CV (%)
(A) In Swiss Webster mouse plasma			
Within-day			
1.00	0.994	99.4	2.91
20.0	20.8	104	5.47
500	497	99.4	1.21
Between-day			
1.00	0.910	91.0	12.7
20.0	20.2	101	1.84
500	507	101	4.24
(B) In BALB/c mouse plasma			
Within-day			
1.00	1.00	100	3.77
20.0	17.7	88.5	13.9
500	540	108	6.47
Between-day			
1.00	1.11	111	8.40
20.0	18.9	94.5	7.79
500	521	104	9.11

bile phase (acetonitrile:0.1 M KH_2PO_4:triethylamine:20 mM sodium dioctylsulfonsuccinate) [26] and, similarly, we found that acridine serves as a suitable internal standard within our assay.

3.2. Assay validation

Fig. 5 shows a typical chromatogram obtained from the HPLC analysis of mouse plasma containing TPT. The mobile phase and detection conditions allow assessment of acridine and TPT without interference. Acridine and TPT are well separated with retention times of 7.4 and 9.0 min, respectively (Fig. 2B). No interfering peaks were found from the assay of blank mouse plasma (Fig. 2A).

The assay was shown to be linear over a concentration range of 1–500 ng/ml, and the linear regression correlation coefficient was greater than 0.999 for all validation runs. The upper limit of linearity was found to be 500 ng/ml; concentrations higher than 500 ng/ml were found to lead to fluorescence output that was lower than that projected by the standard curve. The recoveries of free and total TPT from spiked mouse plasma were within 10% of theoretical values. The within- and between-day CV% were less than 13% for total TPT in Swiss Webster mouse plasma, ≤14% in BALB/c mouse plasma in the absence of anti-TPT antibodies (Table 1A and B), ≤14% in the presence of anti-TPT antibodies (Table 2), and ≤19% in mouse plasma ultrafiltrate (Table 3).

The lower limit of quantitation (LLQ) for TPT in mouse plasma was determined to be 0.02 ng in mouse plasma and mouse plasma ultrafiltrate, which corresponds to a concentration of 1 ng/ml for a 20 μl sample. The sensitivity on column was comparable to those of previously reported HPLC assays (0.01 ng (0.1 ng/ml for 100 μl human plasma sample [16]); 0.01 ng for rat plasma (0.1 ng/ml for 100 μl plasma sample [17]), and 0.02 ng for dog plasma (0.02 ng/ml for 100 μl plasma sample)). The assay of Rosing et al. was more sensitive [24] with a lower limit of quantitation of 0.005 ng (0.05 ng/ml for 100 μl human plasma); however, in our hands, the methods that we report here provided superior results compared to those of Rosing et al. (Fig. 2).

Table 2
Accuracy and precision (CV%) associated with assayed concentrations of quality control samples for total TPT in the presence of anti-TPT antibodies[a]

QC concentration (ng/ml)	Assayed concentration (ng/ml)	Recovery (%)	CV (%)
Within-day			
1.00	1.00	100	10.0
20.0	18.9	94.3	1.86
500	466	93.2	5.85
Between-day			
1.00	0.949	94.9	14.0
20.0	19.3	96.5	8.99
500	539	108	7.67

[a] The assay was validated by preparing QC samples using 20 μl of hyperimmunized BALB/c mouse sera.

Table 3
Accuracy and precision (CV%) associated with assayed concentrations of quality control samples for unbound TPT in mouse plasma[a]

QC concentration (ng/ml)	Assayed concentration (ng/ml)	Recovery (%)	CV (%)
Within-day			
1.00	1.10	110	18.2
20.0	20.9	105	1.99
500	503	101	0.143
Between-day			
1.00	0.980	98.0	16.2
20.0	20.2	101	12.8
500	497	99.3	1.31

[a] The assay was validated using QCs prepared in 20 μl of Swiss Webster mouse plasma ultrafiltrate.

3.3. Assessment of the non-specific adsorption of TPT to Centrifree membrane

As shown in Table 4, the recovery of TPT in 25 mM KH_2PO_4 buffer, pH 3.0 was concentration dependent. Recovery predicted by buffer showed that when TPT concentration higher than 50 ng/ml, over 90% recovery could be assured after ultrafiltration. Recovery was greater than 70% at all concentrations tested.

3.4. Stability of TPT in mouse plasma

TPT was stable in plasma at −80, −20 and 4 °C for up to 24 h (Fig. 6), and TPT was stable in acidified methanol extracts of plasma under all tested conditions (Fig. 7). These results are in consistent with those obtained with human plasma preparations [20]. Based on these results, plasma samples were immediately processed, and stored at −80 °C.

3.5. Pharmacokinetics of TPT in mice

Fig. 8 shows the TPT concentration–time course following an i.p. bolus injection of 10 mg/kg TPT in three Swiss Webster mice. Non-compartmental analysis showed that clearance over bioavailability (CL/*F*) was 3.7 ± 0.4 L/h/kg, in consistent with previous reports in the literature [27,28]. Volume of distribution over bioavailability (*V*/*F*) was 33.0 ± 13.1 L/kg. Half-life ($t_{1/2}$) was 6.3 ± 2.0 h, which was somewhat greater than that reported previously (e.g. $t_{1/2}$ = 1.95 ± 2.77 h [28]).

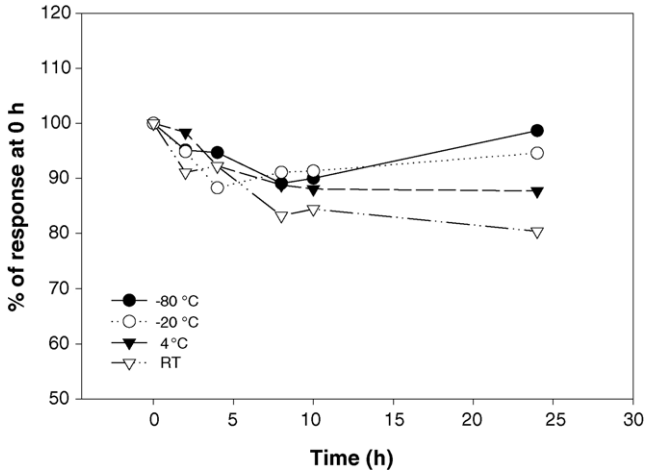

Fig. 6. The effect of storing temperature on TPT stability in mouse plasma for 24 h.

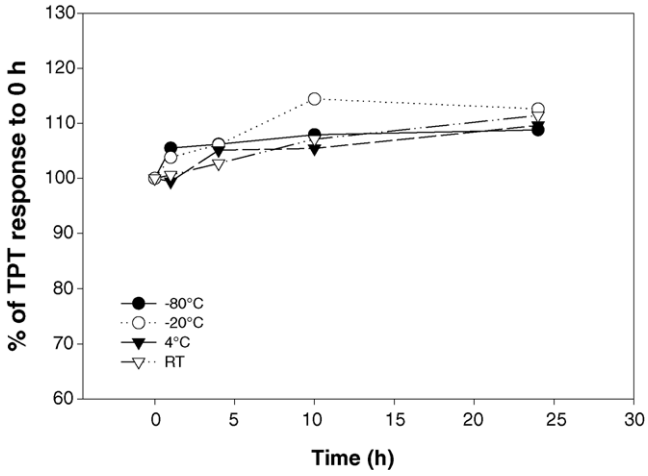

Fig. 7. The effect of storing temperature on TPT stability in mouse plasma acidified methanol extract for 24 h.

Table 4
Recovery of TPT associated with assayed concentrations of QC samples in 25 mM KH_2PO_4 buffer pH 3.0 ultrafiltrate (n = 3)

QC concentration (ng/ml)	Mean assayed concentration (ng/ml)	Recovery (%)	CV (%)
500	446	89.2	7.41
50.0	48.4	96.7	1.97
20.0	14.9	74.7	0.773
10.0	7.13	71.3	1.62

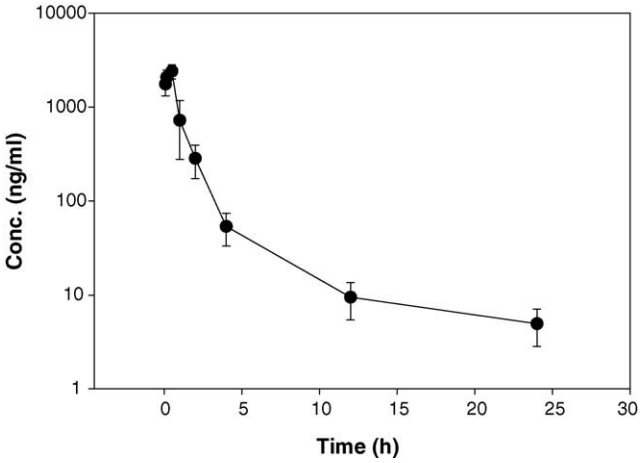

Fig. 8. The TPT concentration–time course following i.p. bolus injection of 10 mg/kg TPT in Swiss–Webster mice ($n = 3$).

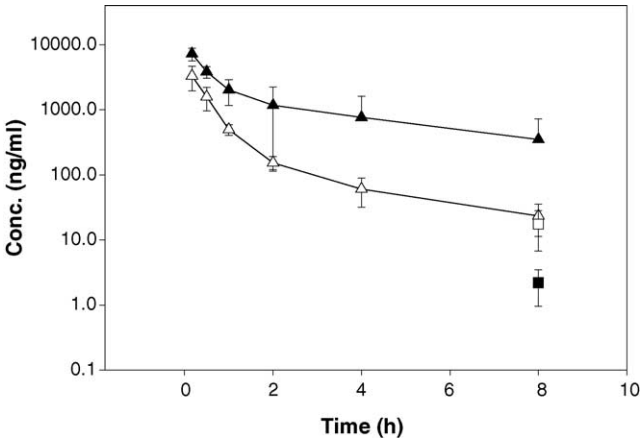

Fig. 9. Total TPT concentration–time profile in BALB/c hyperimmunized (closed triangle) and control mice (open triangle) and free TPT concentration at 8 h for BALB/c hyperimmunized (closed square) and control mice (open square) following an i.v. bolus of 10 mg/kg ($n = 3$ for each group).

3.6. Verification of antibody binding in hyperimmunized BALB/c plasma via protein-G adsorption

To verify the presence of anti-TPT immunoglobulin in plasma obtained from hyperimmunized mice, TPT concentrations were determined in diluted plasma samples before and after sample treatment via ultrafiltration and protein-G affinity adsorption. Our data (Table 5) showed that in immunized animals, over 98% TPT was removed by treatment with a protein-G affinity column (TPT concentration in the eluate was less than 2% of the concentration before protein-G treatment). No significant reductions to TPT concentrations were observed following application of the protein-G treatment to TPT samples prepared from non-immunized mice. As such, in combination, these data provide strong support to our hypothesis that the present immunization strategy leads to the development of an anti-TPT antibody response.

Due to the high affinity of protein-G to IgG, it is expected that protein-G treatment would remove TPT that was bound to IgG. On the other hand, ultrafiltration would be expected to remove TPT that is bound to all plasma proteins (e.g. albumin, IgG, other immunoglobulins, etc.). Consistent with these arguments, we found that the free fraction obtained by using ultrafiltration was less than that obtained by using protein-G column (Table 5), and SDS–PAGE analysis did not reveal the presence of IgG bands in plasma that was treated with protein-G or ultrafiltrated (data not shown). We observed only small differences between TPT concentrations determined following ultrafiltration and following protein-G treatment, and this finding is consistent with previous reports that show little TPT binding to plasma proteins (e.g. less than ~20% of TPT is reported to be bound to plasma protein in human studies [10,23]).

3.7. Pharmacokinetics of TPT in the presence of anti-TPT antibodies

The TPT concentration–time profiles following an i.v. administration of 10 mg/kg in hyperimmunized and control BALB/c mice are shown in Fig. 9. As shown, the presence of anti-TPT antibodies dramatically altered the time course of TPT disposition in mice. The area under the concentration–time curve was found to be 12.0 ± 8.3 mg/L × h versus 2.6 ± 0.7 mg/L × h for the hyperimmunized and control animals, respectively. The unbound TPT concentration at 8 h for the control animals is much higher than that of immunized animals. Taken collectively, these data suggested that the presence of anti-TPT antibodies could alter the disposition of TPT, leading to an increase in to-

Table 5
Verification of antibody binding in hyperimmunized BALB/c plasma via protein-G adsorption

Plasma	Plasma dilution	Total TPT (ng/ml)[a,b]	Protein-G adsorption		Ultrafiltration	
			Unbound TPT (ng/ml)	f_u	Unbound TPT (ng/ml)	f_u
Immunized	6×	68.44 (1.09)	1.04 (0.11)	0.015	0.85 (0.12)	0.012
	15×	66.55 (2.23)	17.21 (0.43)	0.259	15.38 (1.26)	0.231
	60×	65.82 (2.28)	59.68 (1.05)	0.907	53.17 (4.51)	0.808
Blank	6×	70.14 (2.00)	68.92 (1.96)	0.983	NA	
	15×	66.06 (2.57)	63.35 (2.44)	0.959	NA	
	60×	66.21 (3.69)	61.51 (3.27)	0.929	NA	

[a] The nominal total TPT concentration for these experiments was 66.67 ng/ml.
[b] Data are presented as mean concentrations with the standard deviation in parentheses.

tal TPT systemic exposure while decrease free TPT systemic exposure, as hypothesized.

4. Conclusion

In conclusion, a sensitive and simple HPLC method for analysis of the total and free TPT concentration in mouse plasma in the presence and absence of anti-TPT antibodies has been developed and validated. To our knowledge, this is the first validated HPLC method for analysis of TPT in mouse plasma. This assay was shown to have sufficient specificity, accuracy, precision, and sensitivity for our proposed pharmacokinetic studies in mice. This assay will allow us to evaluate the effect of anti-TPT antibodies on the pharmacokinetics of TPT in murine ovarian cancer models.

References

[1] W.P. McGuire, W.J. Hoskins, M.F. Brady, P.R. Kucera, E.E. Partridge, K.Y. Look, D.L. Clarke-Pearson, M. Davidson, N. Engl. J. Med. 334 (1996) 1.
[2] A. du Bois, Eur. J. Cancer 37 (Suppl. 9) (2001) 1.
[3] D.S. Alberts, P.Y. Liu, E.V. Hannigan, R. O'Toole, S.D. Williams, J.A. Young, E.W. Franklin, D.L. Clarke-Pearson, V.K. Malviya, B. DuBeshter, N. Engl. J. Med. 335 (1996) 1950.
[4] R.L. Dedrick, C.E. Myers, P.M. Bungay, V.T. DeVita Jr., Cancer Treat. Rep. 62 (1978) 1.
[5] J. Balthasar, H.L. Fung, J. Pharmacol. Exp. Ther. 268 (1994) 734.
[6] J.P. Balthasar, H.L. Fung, J. Pharm. Sci. 85 (1996) 1035.
[7] E.D. Lobo, D.M. Soda, J.P. Balthasar, J. Pharm. Sci. 92 (2003) 1665.
[8] M. Markman, Semin. Oncol. 24 (1997) S5.
[9] V.M. Herben, W.W. ten Bokkel Huinink, J.H. Beijnen, Clin. Pharmacokinet. 31 (1996) 85.
[10] Y.H. Hsiang, L.F. Liu, Cancer Res. 48 (1988) 1722.
[11] Y.H. Hsiang, R. Hertzberg, S. Hecht, L.F. Liu, J. Biol. Chem. 260 (1985) 14873.
[12] S.C. Plaxe, R.D. Christen, J. O'Quigley, P.S. Braly, J.L. Freddo, E. McClay, D. Heath, S.B. Howell, Invest. New Drugs 16 (1998) 147.
[13] L.S. Hofstra, A.M. Bos, E.G. Vries, A.G. Zee, J.H. Beijnen, H. Rosing, N.H. Mulder, J.G. Aalders, P.H. Willemse, Br. J. Cancer 85 (2001) 1627.
[14] J.H. Beijnen, B.R. Smith, W.J. Keijer, R. van Gijn, W.W. ten Bokkel Huinink, L.T. Vlasveld, S. Rodenhuis, W.J. Underberg, J. Pharm. Biomed. Anal. 8 (1990) 789.
[15] W.J. Loos, G. Stoter, J. Verweij, J.H. Schellens, J. Chromatogr. B Biomed. Appl. 678 (1996) 309.
[16] H. Rosing, D.M. van Zomeren, E. Doyle, W.W. ten Bokkel, J.H. Schellens, A. Bult, J.H. Beijnen, J. Chromatogr. B Biomed. Sci. Appl. 727 (1999) 191.
[17] H. Rosing, E. Doyle, J.H. Beijnen, J. Pharm. Biomed. Anal. 15 (1996) 279.
[18] C. Kollmannsberger, K. Mross, A. Jakob, L. Kanz, C. Bokemeyer, Oncology 56 (1999) 1.
[19] J.G. Wall, H.A. Burris III, D.D. Von Hoff, G. Rodriguez, R. Kneuper-Hall, D. Shaffer, T. O'Rourke, T. Brown, G. Weiss, G. Clark, et al., Anticancer Drugs 3 (1992) 337.
[20] L.B. Grochow, E.K. Rowinsky, R. Johnson, S. Ludeman, S.H. Kaufmann, F.L. McCabe, B.R. Smith, L. Hurowitz, A. DeLisa, R.C. Donehower, et al., Drug Metab. Dispos. 20 (1992) 706.
[21] L.J. van Warmerdam, J. Verweij, J.H. Schellens, H. Rosing, B.E. Davies, M. de Boer-Dennert, R.A. Maes, J.H. Beijnen, Cancer Chemother. Pharmacol. 35 (1995) 237.
[22] H. Rosing, V.M. Herben, D.M. van Gortel-van Zomeren, E. Hop, J.J. Kettenes-van den Bosch, W.W. ten Bokkel Huinink, J.H. Beijnen, Cancer Chemother. Pharmacol. 39 (1997) 498.
[23] H. Rosing, D.M. van Zomeren, E. Doyle, A. Bult, J.H. Beijnen, Anticancer Drugs 9 (1998) 587.
[24] H. Rosing, E. Doyle, B.E. Davies, J.H. Beijnen, J. Chromatogr. B Biomed. Appl. 668 (1995) 107.
[25] F. Bai, M.N. Kirstein, S.K. Hanna, L.C. Iacono, B. Johnston, C.F. Stewart, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 784 (2003) 225.
[26] D. Subramanian, E. Kraut, A. Staubus, D.C. Young, M.T. Muller, Cancer Res. 55 (1995) 2097.
[27] W.C. Zamboni, P.J. Houghton, R.K. Johnson, J.L. Hulstein, W.R. Crom, P.J. Cheshire, S.K. Hanna, L.B. Richmond, X. Luo, C.F. Stewart, J. Pharmacol. Exp. Ther. 284 (1998) 89.
[28] M. De Cesare, F. Zunino, S. Pace, C. Pisano, G. Pratesi, Eur. J. Cancer 36 (2000) 1558.