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ORIGINAL ARTICLE

Plasma pharmacokinetics and oral bioavailability of the 3,4,5,6-tetrahydrouridine (THU) prodrug, triacetyl-THU (taTHU), in mice

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Abstract
Purpose Cytidine drugs, such as gemcitabine, undergo rapid catabolism and inactivation by cytidine deaminase (CD). 3,4,5,6-tetrahydrouridine (THU), a potent CD inhibitor, has been applied preclinically and clinically as a modulator of cytidine analogue metabolism. However, THU is only 20% orally bioavailable, which limits its preclinical evaluation and clinical use. Therefore, we characterized THU pharmacokinetics after the administration to mice of the more lipophilic pro-drug triacetyl-THU (taTHU).
Methods Mice were dosed with 150 mg/kg taTHU i.v. or p.o. Plasma and urine THU concentrations were quantitated with a validated LC–MS/MS assay. Plasma and urine pharmacokinetic parameters were calculated non-compartmentally and compartmentally.
Results taTHU did not inhibit CD. THU, after 150 mg/kg taTHU i.v., had a 235-min terminal half-life and produced plasma THU concentrations >1 μg/mL, the concentration shown to inhibit CD, for 10 h. Renal excretion accounted for 40–55% of the i.v. taTHU dose, 6–12% of the p.o. taTHU dose. A two-compartment model of taTHU generating THU fitted the i.v. taTHU data best. taTHU, at 150 mg/kg p.o., produced a concentration *versus* time profile with a plateau of approximately 10 μg/mL from 0.5–2 h, followed by a decline with a 122-min half-life. Approximately 68% of i.v. taTHU is converted to THU. Approximately 30% of p.o. taTHU reaches the systemic circulation as THU.
Conclusions The availability of THU after p.o. taTHU is 30%, when compared to the 20% achieved with p.o. THU. These data will support the clinical studies of taTHU.

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Keywords Tetrahydrouridine · THU · Bioavailability · Metabolism · Mouse · Cytidine deaminase

Introduction

Important anticancer agents, such as cytosine arabinoside (Ara-C), gemcitabine, decitabine, and 5-azacytidine, are cytidine analogues [22]. Classically, nucleosides have been developed as cytotoxic drugs. More recently, several cytidine analogues have been licensed (decitabine and 5-azacytidine) or are in clinical trials (5-fluoro-2′-deoxycytidine), based on their ability to inhibit methylation of DNA [5, 15]. Most cytidine analogues are metabolized and inactivated by cytidine deaminase (CD) (EC 3.5.4.5) [12], and CD-mediated deamination is considered the main cause for the short in vivo half-lives of these drugs [9, 18, 24, 31]. Consequently, because of their chemical and pharmacokinetic properties, most of the current nucleoside cytidine drugs require parenteral administration [26]. Oral (p.o.) dosing would require passage of the drug across the gastrointestinal tract and through the liver, each of which has high levels of CD. This would limit the amount of drug that would reach the systemic circulation and result in low p.o. bioavailability.

3,4,5,6-tetrahydrouridine (THU) is a potent CD inhibitor that has been applied preclinically and clinically as a modulator of cytidine analogue metabolism [6, 9, 17, 18, 24, 25, 27, 31, 37]. By potently inhibiting CD, THU may prolong the half-life of i.v. cytidine analogues, and by inhibiting CD in the gut and liver, THU can increase the bioavailability of p.o. administered cytidine analogues [5, 9, 18, 24, 29]. This latter property may enable convenient, chronic p.o. dosing of cytidine analogues, which could produce sustained, adequate plasma concentrations and permit chronic use for durations that are impractical with i.v. formulations.

Although THU has been applied as a modifier of cytidine metabolism in several clinical trials [6, 24, 25, 27, 31, 37], lack of suitable analytical methodology had prevented adequate characterization of THU pharmacokinetics. We previously developed a specific and sensitive analytical method for the quantitation of THU [32] and used it to demonstrate the p.o. bioavailability of THU in mice to be approximately 20% [3], which was sufficient to produce THU plasma concentrations adequate for inhibition of CD. However, improvement in the bioavailability of THU could result in less frequent dosing and more extensive inhibition of CD in compartments other than plasma. Better p.o. bioavailability might be achieved by delivering THU as a prodrug that is more lipophilic than THU and capable of direct transmembrane absorption, bypassing capacity-limited intestinal transporters. Triacetylation is an approach that has successfully been used previously to improve the p.o. bioavailability of 6-azauridine [7]. We have now characterized murine THU plasma and urine pharmacokinetics after i.v. or p.o. administration of triacetyl-THU (taTHU).

Materials and methods

Chemicals and reagents

3,4,5,6-Tetrahydrouridine, 2′,3′,5′-triacetyl-3,4,5,6-tetrahydrouridine, and gemcitabine were provided by the Developmental Therapeutics Program, National Cancer Institute (Rockville, MD). [D_4]-THU internal standard was synthesized by Ash Stevens Inc. (Detroit, MI) and provided by the Developmental Therapeutics Program, National Cancer Institute. All solvents were obtained from Fisher Chemicals (Fair Lawn, NJ). Formic acid was obtained from Sigma–Aldrich (St. Louis, MO). All chemicals were of analytical grade. Water was purified using a Q-gard® 1 Gradient Milli-Q system (18.2 MΩ cm, Millipore, Billerica, MA). Control murine plasma was obtained from Lampire Biological Laboratories (Pipersville, PA).

Animals

Specific-pathogen-free, adult CD_2F_1 male mice were purchased from Taconic (Germantown, NY). Mice were allowed to acclimate to the University of Pittsburgh Cancer Institute Animal Facility for ≥1 week before being used. To minimize infection, mice were maintained in micro-isolator cages in a separate room and handled in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and on a protocol approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Ventilation and airflow were set to 12 changes per h. Room temperatures were regulated at 22 ± 1°C, and the rooms were kept on automatic 12-h light/dark cycles. Mice received Prolab ISOPRO RMH 3000 Irradiated Lab Diet (PMI Nutrition International, St. Louis, MO) and water ad libitum, except on the evening before dosing, when all food was removed. Mice were 6–8 weeks old and weighed approximately 20 g at the time of dosing. Sentinel animals were maintained in the rooms housing study mice and assayed at 3-month intervals for specific murine pathogens by mouse antibody profile testing (Charles River, Boston, MA). Sentinel animals remained free of specific pathogens, indicating that the study mice were pathogen free.

Ability of taTHU to inhibit CD

Because the ability of taTHU to inhibit CD was unknown, we assessed the effect of taTHU on the deamination of

gemcitabine to dFdU by recombinant human CD [16]. Briefly, incubations were performed in duplicate at 37°C in 50 mM Tris buffer, pH 7.5 with 2 mM dithiothreitol. The negative control contained 500 μM of taTHU, gemcitabine, and THU, but no CD. The positive control contained 10 μL of CD and 500 μM of gemcitabine. A second positive control contained 10 μL of CD, 500 μM gemcitabine, and 500 μM THU. The test solution contained 10 μL of CD, 500 μM gemcitabine, and 500 μM taTHU. The final incubation volume was 250 μL. At 5, 10, 15, 20, 30, and 60 min after starting the incubation, aliquots of 10 μL were removed and added to 100 μL of ethyl acetate, thereby quenching the reaction. After dilution with 100 μL of plasma, concentrations of dFdU were quantitated with a previously described assay [4] that had been modified by using [$^{15}N_2$, ^{13}C]-dFdU as internal standard (Toronto Research Chemicals Inc., North York, ON, Canada). At the last time point, we also analysed the sample for any THU produced from taTHU by CD. The protein content of the CD solutions was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL), according to the manufacturer's directions.

Stability of taTHU in dosing solution and in murine plasma

taTHU dosing solution (15 mg/mL taTHU in 5% dextrose) was analysed for THU content after 16 h at room temperature. In addition, 10 μg/mL taTHU was added to mouse plasma and kept on ice or at room temperature, and samples were taken at 0, 1, 2, and 4 h. Samples were assayed for THU [32] and taTHU (semi-quantitative). We also assessed the ability of mouse and human blood to hydrolyse taTHU to THU. Whole blood containing 150 μg/mL taTHU was incubated at 37°C in a shaking water bath. Plasma was obtained by centrifugation of whole blood, and THU was quantitated as described below.

Plasma disposition

To investigate the plasma pharmacokinetics of THU after the administration of taTHU, mice were dosed with 150 mg/kg taTHU (equimolar to 100 mg/kg THU, the dose used in previous studies) dissolved in 5% dextrose (Baxter, Deerfield, IL) (dosing solution 15 mg/mL; 0.01 mL/g). Doses were delivered i.v. by lateral tail vein injection or p.o. by oral gavage.

Mice (three per time point) were euthanized with CO_2 before and at 5, 10, 15, 30, 45, 60, 120, 240, 420, 960, and 1,440 min after dosing. Blood was collected by cardiac puncture into heparinized syringes and centrifuged for 5 min at 13,000×*g* to obtain plasma. Plasma samples were stored at −70°C until analysis.

THU concentrations in plasma were quantitated with a previously developed and validated HILIC LC–MS/MS assay [32]. The assay fulfilled the FDA criteria for bioanalytical method validation [34] and was accurate and precise in the concentration range of 0.2–50 μg/mL. The linear range was extended downwards to 0.05 μg/mL by adding 0.05 and 0.1 μg/mL calibration points, and a 0.1 μg/mL QC sample exhibited acceptable performance. We also added an MRM channel for taTHU (*m/z* 374.7 to 259, corresponding to the molecular ion $[M + H]^+$ fragmenting to triacetyl ribose).

Urinary excretion

To assess urinary excretion of THU, mice scheduled for euthanasia at 16 and 24 h after dosing were kept in metabolic cages after administration of taTHU. Urine was collected on ice at 6 h and 24 h after dosing for the 24 h mice, and after 16 h for the 16 h mice. At the end of the collection period, cages were washed with 15 mL of water. Quantitation of THU in urine and cage wash was accomplished by diluting (at least tenfold) an aliquot of each sample with control plasma and analysing that diluted sample with the HILIC LC–MS/MS assay used for plasma samples [32]. In addition, urine samples were treated chemically to hydrolyse mono-, di-, and triacetyl-THU to THU. Therefore, we used a chemical hydrolysis approach. Specifically, urine aliquots of 200 μL were mixed with 20 μL of 1 N sodium hydroxide and incubated in a vortex mixer for 1 h at room temperature. The reaction was quenched by the addition of 5 μL of 2 M sulphuric acid, and THU was quantitated as described above [32]. THU was stable under these conditions. Longer incubations (2 h) or use of more base did not increase the conversion of taTHU to THU. The conversion efficiency of taTHU to THU was established by hydrolysing urine samples containing 5 to 500 μg/mL taTHU (7 concentrations total in duplicate curves, on 3 separate occasions) and quantitating the resulting THU with a THU standard calibration curve. The hydrolytic conversion of taTHU to THU in urine was consistent and determined to be 59.7% (3.4% CV, $R^2 > 0.9959$).

Pharmacokinetic analysis

The maximum THU plasma concentration (C_{max}) and the time to reach it (T_{max}) were determined by visual inspection of the plasma concentration *versus* time data. Other pharmacokinetic parameters for THU after i.v. or p.o. dosing were calculated non-compartmentally using PK Solutions 2.0 (Summit Research Services, Montrose, CO http://www.summitPK.com). The area under the plasma concentration *versus* time curve (AUC) of THU was calculated with the linear trapezoidal rule. Renal clearance of THU was calculated by dividing the amount of THU excreted by the plasma AUC_{0-inf} of THU.

Plasma THU concentration *versus* time data were also analysed compartmentally using the ADAPT 5 software for pharmacokinetic/pharmacodynamic systems analysis [8]. The maximum likelihood option in ADAPT 5 was used for all estimations, and model selection was based on Akaike's information criterion (AIC) [1]. The starting point for the compartmental modelling was our previously developed model for THU after i.v. and p.o. administration of THU [3]. As a rule, upon adding a new set of data and parameters, initially all known parameters were fixed, and initial values for new parameters were determined. Next, all non-peripheral parameters were released, and then, final parameter values were established by allowing all parameters to float.

Results

Analytical

Triacetyl-THU (taTHU) eluted very early (1.8 min), which is consistent with its lower polarity relative to THU. taTHU did not cross-talk into the THU MRM channel. Because of the very short retention of taTHU and the lack of an isotopic taTHU internal standard, we decided not to amend the assay for THU to include the quantitation of taTHU or the mono- and diacetyl-THU intermediates (Fig. 1). However, we did include the taTHU MRM channel in the mass spectrometric method for semiquantitative purposes.

In vitro in mouse plasma, the taTHU signal decreased monoexponentially to 50% of its starting value over 4 h. No THU was detected in samples obtained during the first 2 h, and only the 4-h sample gave a THU response of approximately five times the background noise, i.e. much lower than the limit of quantitation. A 16-h incubation of the 15 mg/mL taTHU dosing solution in 5% dextrose at room temperature did not produce any THU. Incubation of taTHU at 150 μg/mL in murine whole blood at 37°C resulted in a rapid generation of THU at a rate of approximately 160 ng/mL/min (minimum of three time points over 4 h). A similar incubation of taTHU in human blood resulted in the generation of THU at a rate of 200 ng/mL/min.

Ability of taTHU to inhibit CD

Recombinant human CD (0.22 μg/μL) resulted in the production of dFdU at a rate of 191 ng/mL/min, see Fig. 2. THU (500 μM) completely abrogated such deamination. taTHU (500 μM) decreased the deamination of gemcitabine to dFdU by 20% to 152 ng/mL/min. This was considered a negligible effect, especially given the equimolar concentrations of gemcitabine and taTHU. We did not detect any THU after incubation of taTHU with CD. No generation of dFdU was observed upon incubation of taTHU, THU, and gemcitabine without CD.

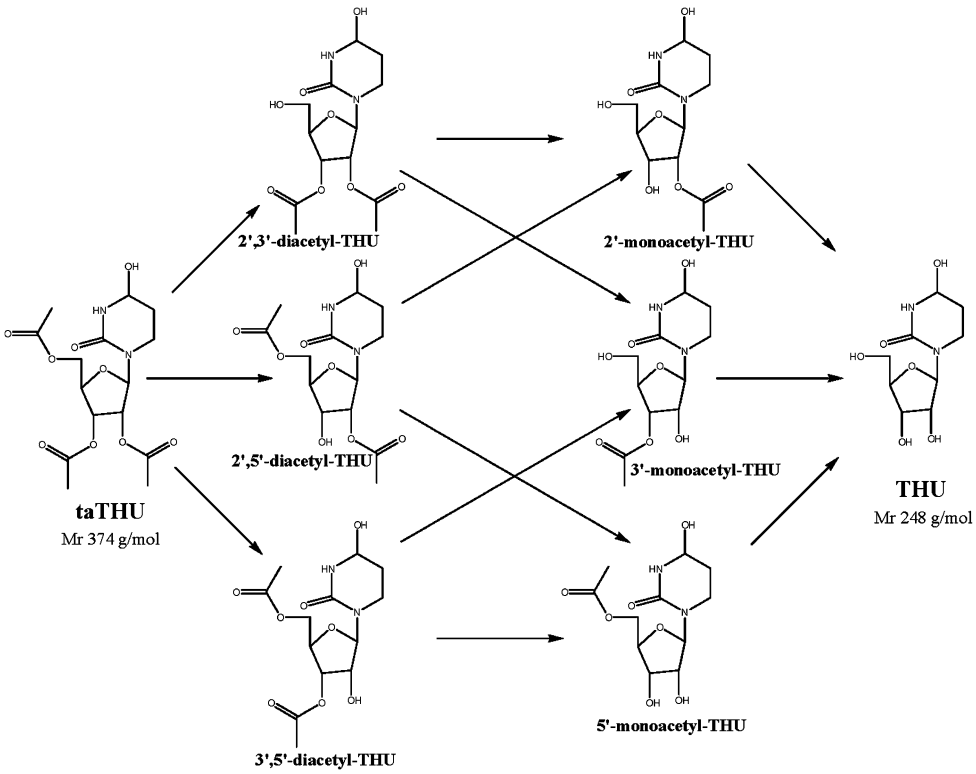

Fig. 1 Chemical structure of 3,4,5,6-tetrahydrouridine (THU), its prodrug 2′,3′,5′-triacetyl-3,4,5,6-tetrahydrouridine (taTHU), and all the possible metabolic intermediates: 2′,3′-diacetyl-THU, 2′,5′-diacetyl-THU, 3′,5′-diacetyl-THU, 2′-monoacetyl-THU, 3′-monoacetyl-THU, and 5′-monoacetyl-THU

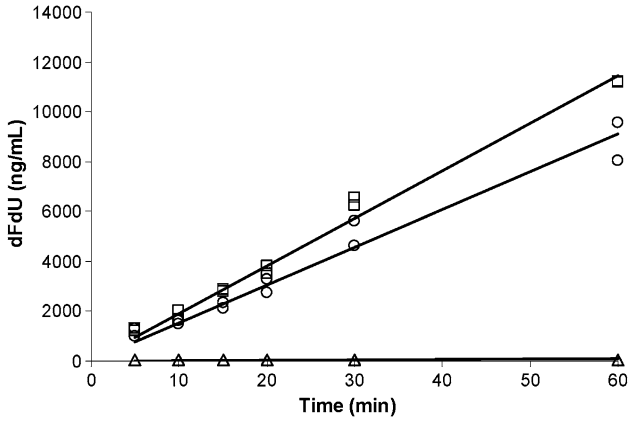

Fig. 2 Production of dFdU after incubation of 500 μM (131 μg/mL) dFdC at 37°C with: recombinant human CD (*white square*, 191 ng/mL/min, R^2 0.989); CD and 500 μM taTHU (*white circle*, 152 ng/mL/min, R^2 0.965); and CD and 500 μM THU (*white triangle*)

Plasma disposition

No taTHU could be detected in plasma samples obtained after i.v. or p.o. administration of taTHU to mice. The plasma concentration *versus* time data profiles of THU after administration of 150 mg/kg taTHU i.v. or p.o. are shown in Fig. 3a–d (with compartmental model predictions) as are the previously published THU plasma concentration *versus* time profiles produced by i.v. or p.o. administration of the equimolar dose of 100 mg/kg THU [3]. Non-compartmental pharmacokinetic parameters of THU after administration of taTHU and those previously published after administration of THU [3] are displayed in Table 1. After i.v. administration of taTHU, THU C_{max} was observed at the first time point sampled (5 min, Fig. 3a). Thereafter, plasma concentrations declined multi-exponentially with a terminal half-life of more than 200 min. THU could be detected until 960 min after the i.v. taTHU dose. After p.o. taTHU, THU plasma concentrations rose rapidly during the first 30 min, remained relatively constant for approximately 2 h, and then decreased (Fig. 3b). The T_{max} after p.o. dosing of taTHU was between 30 and 120 min but could not be determined more accurately because of the relatively flat THU concentration *versus* time profile. Based on the THU AUC values, the THU bioavailability of i.v. taTHU relative to i.v. THU was 68%, and the THU bioavailability of p.o. taTHU relative to i.v. THU was 30% (Table 1).

Urinary excretion

Urinary excretion of THU represented 25.6–35.2% of the dose after i.v. administration of taTHU and represented 5.2–11.2% of the dose after p.o. administration of taTHU (Table 2). This corresponded to a THU renal clearance of 1.5–4.6 mL/min/kg. Urinary excretion of analytes capable of generating THU represented an additional 14.1–19.4% of the dose after i.v. administration of taTHU but only an additional 0.42–0.71% of the dose after p.o. administration of taTHU.

Compartmental pharmacokinetic analysis

We also performed compartmental analyses on the THU plasma concentration *versus* time data (lines in Fig. 3a–d). The starting model consisted of compartments 1, 2, and 3 and the previously published data associated with a 100 mg/kg i.v. THU dose [3]. Compartment 4 was added to represent taTHU in the central compartment after i.v. administration of taTHU. The hydrolysis of all three acetyl moieties from a fraction (F_{conv}) of the dose of taTHU was modelled as one linear step into compartment 1 (THU central compartment), with the remainder (1-F_{conv}) of taTHU lost by other means. The resulting model (AIC 605; R^2 0.959) did not adequately fit to the THU data after i.v. taTHU administration. The model overestimated the early points and underestimated the later points. Introduction of a concatenary compartment between compartments 4 and 1, with loss either from compartment 4 or the concatenary compartment, more reflective of the stepwise chemical conversions from taTHU to THU, did improve the fit somewhat (AIC 500; R^2 0.972). Ultimately, a mammillary compartment 5 was added to compartment 4, which effected an even better fit of the model (AIC 459; R^2 0.976). Next, we attempted to model the hydrolysis steps explicitly between compartments 4 and 1, according to the scheme in Fig. 1, with an additional loss rate constant for each intermediate. To allow the required addition of 6 additional compartments while minimizing the complexity added to the model, we assumed: (1) that each hydrolysis step had the same rate constant and (2) that the rate constant of other clearance pathways was identical for taTHU and all di- and monoacetyl-THU intermediates. In this manner, the total number of parameters did not increase. The resulting model was not able to capture the rapid dynamics of the observed increase in THU concentrations after i.v. taTHU administration. Imposing higher values for the hydrolysis rate constant reduced the discrepancy (in effect creating a rapid bolus-like generation of THU in compartment 1) but caused the model to miss the later THU data points after i.v. THU administration and did not result in a significant improvement in the fit (AIC 469; R^2 0.972).

Subsequently, we added compartment 6 to represent p.o. administration of taTHU, with a rate of absorption into compartment 4, and a fractional loss of p.o. taTHU by other means. The model fit the data well (AIC 567; R^2 0.978). However, the urine data suggested that no p.o. taTHU is absorbed intact into the systemic circulation before conversion

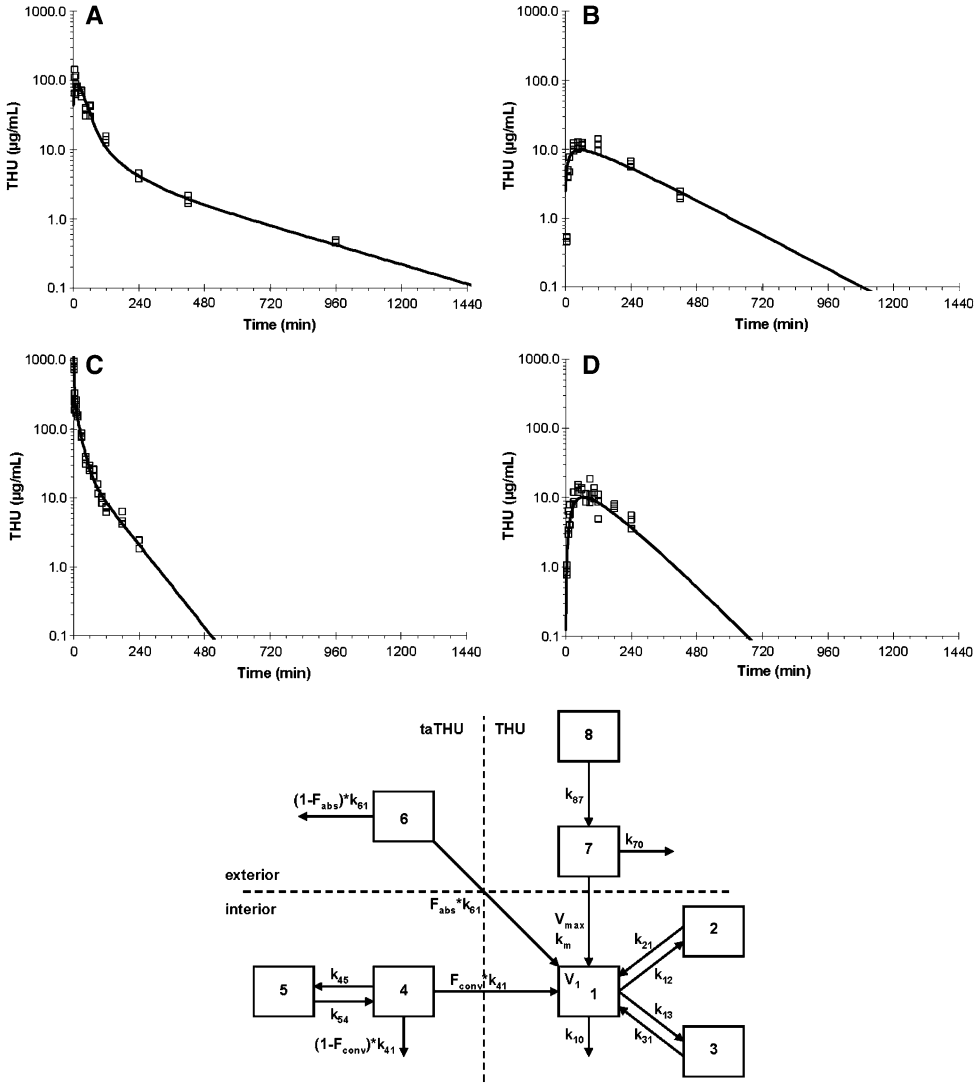

Fig. 3 Predictions (*solid lines*) of the final model and actual data (*white square*) after administration of 150 mg/kg taTHU i.v. (**a**) or p.o. (**b**) or 100 mg/kg THU i.v (**c**) or p.o. (**d**) to mice, using a compartmental model (**e**), constructed to be fitted to THU mouse plasma concentration data obtained after administration of 150 mg/kg taTHU i.v or p.o. in combination with previously published data [3] of THU administered to mice at 100 mg/kg i.v., 300 mg/kg p.o., 100 mg/kg p.o. or 30 mg/kg p.o. The entire p.o. THU dose is delivered to compartment 8 and transferred to compartment 7 (k_{87}), from which it is either eliminated (k_{70}, never absorbed) or transferred into the central compartment V_1 via a non-linear rate (V_{max}, K_m). The i.v. THU dose is delivered to V_1 as a bolus. Distribution takes place to and from compartments 2 and 3 by their respective rate constants (k_{12}, k_{21}, k_{13}, k_{31}), and elimination from V_1 is determined by k_{10}. The i.v. taTHU dose is delivered as a bolus to compartment 4, from which it can distribute into compartment 5 according to the respective rate constants (k_{45}, k_{54}). The p.o. taTHU dose is delivered as a bolus into compartment 6, from which only a fraction (F_{abs}) reaches compartment 1 at a specific rate (F_{abs}*k_{61}), while the rest is lost (unabsorbed). A fraction of taTHU from compartment 4 (F_{conv}) is converted to THU at a specific rate (F_{conv}*k_{41}; with correction for difference in molecular weights), while the remainder is lost

to THU. The urine data provide compelling evidence that any taTHU that is absorbed from the gastrointestinal tract is essentially totally converted to THU. This alternate model structure is represented by a transfer and conversion of taTHU in compartment 6 to THU in compartment 1, instead of through compartment 4. The resulting model appeared somewhat less suitable than the one with taTHU absorption into compartment 4 because it underestimated the higher THU data points after p.o. taTHU (AIC 618; R^2 0.976). However, because of the compelling urine data, we settled on the model with direct transfer of p.o. taTHU into THU in compartment 1.

Finally, the model was reconnected with compartments 7 and 8 (Fig. 3e), which correspond to previously published information related to p.o. THU administration at 30, 100, and 300 mg/kg [3]. The final values of the pharmacokinetic parameters of the resulting model (AIC 1065; R^2 0.974) are listed in Table 3. Most importantly, the fraction of i.v.

Table 1 Non-compartmental pharmacokinetic parameters of THU after i.v. or p.o. administration to male CD_2F_1 mice of 150 mg/kg taTHU i.v. or p.o., or 100 mg/kg THU i.v. or p.o.

Parameter	Route (dose, mg/kg)			
	taTHU i.v. (150)	taTHU p.o. (150)	THU i.v. (100)	THU p.o. (100)
C_{max} (μg/mL)	106	11.8	814	13.7
T_{max} (min)	5	60	2	45
Half-life (min)	235	122	73	85
AUC_{0-240} (mg/mL•min)	6.21	2.25	10.9	2.01
AUC_{0-inf} (mg/mL•min)[a]	7.55	3.36	11.1	2.57
Vd/F_1 (L/kg)	4.49	5.26	0.949	4.78
Cl/F_1 (mL/min/kg)	13.2	29.8	9.02	38.9
F_2 (%)	68.0	30.3	–	23.2

THU data after administration of THU were published previously [3]

Vd volume of distribution; F_1: 1) fraction of taTHU that is converted to THU for the i.v. taTHU study, 2) fraction of taTHU that reaches the systemic circulation and is converted to THU for the p.o. taTHU study, 3) p.o. bioavailability for the p.o. THU study; *Cl* total body clearance; F_2 THU plasma exposure relative to plasma exposure after i.v. THU administration

[a] The percentage of the AUC_{0-inf} that was extrapolated from the observed AUC ranged from 1.1–22%

Table 2 0–16 and 0–24 h urinary excretion of THU after administration to male CD_2F_1 mice of 150 mg/kg taTHU i.v. or p.o., or 100 mg/kg THU i.v. or p.o.

Time (h)	Parameter	Route (dose, mg/kg)			
		taTHU i.v. (150)	taTHU p.o. (150)	THU i.v. (100)	THU p.o. (100)
0–16	taTHU dose (mg/mouse)	3.26	3.23	–	–
	THU dose (mg/mouse)	–	–	2.17	2.22
	Dose (mg THU-equiv/mouse)	2.16	2.14	2.17	2.22
	taTHU Excreted (mg/mouse)[a]	0.458	0.014	–	–
	THU Excreted (mg/mouse)	0.552	0.110	1.20	0.351
	THU Cl_{renal} (mL/min/kg)	3.37	1.52	5.02	6.15
	taTHU Excreted (% dose)[a]	14.1	0.421	–	–
	THU Excreted (% dose)	25.6	5.2	55.4	15.8
	Total excreted (% dose)	39.7	5.6	55.4	15.8
0–24	taTHU dose (mg/mouse)	3.22	3.10	–	–
	THU dose (mg/mouse)	–	–	2.18	2.18
	Dose (mg THU-equiv/mouse)	2.14	2.05	2.18	2.18
	taTHU Excreted (mg/mouse)[a]	0.624	0.022	–	–
	THU Excreted (mg/mouse)	0.751	0.229	0.778	0.233
	THU Cl_{renal} (mL/min/kg)	4.63	3.30	3.23	4.16
	taTHU Excreted (% dose)[a]	19.4	0.714	–	–
	THU Excreted (% dose)	35.2	11.2	35.6	10.7
	Total excreted (% dose)	54.6	11.9	35.6	10.7

THU data after administration of THU were published previously [3]

[a] All the THU generated after hydrolysis of urine is assumed to be generated from taTHU

taTHU that is converted to THU in the mice is approximately 69% (F_{conv}), while the fraction of p.o. taTHU that gets absorbed and converted to THU is approximately 30% (F_{abs}). Predictions of the model, shown in Fig. 3a–d, suggest a more prolonged plasma THU exposure after taTHU administration than after administration of THU.

Discussion

THU, in combination with cytidine analogues, has been studied preclinically and clinically [6, 9, 17, 18, 24, 25, 27, 31, 37]. Co-dosing of THU with cytidine analogues could improve the p.o. absorption of a cytidine analogue into the

Table 3 Estimated values for the parameters of the compartmental model shown in Fig. 3

Parameter/model	Estimate (% CV)	95% CI
V_1 (L/kg)	0.042 (48)	0.0015–0.082
k_{10} (1/min)	0.239 (40)	0.046–0.43
k_{12} (1/min)	0.407 (43)	0.057–0.78
k_{21} (1/min)	0.139 (21)	0.081–0.20
k_{13} (1/min)	0.0597 (46)	0.0047–0.11
k_{31} (1/min)	0.0151 (19)	0.0094–0.021
k_{41} (1/min)	0.0372 (15)	0.026–0.049
F_{conv}	0.696 (10)	0.55–0.84
k_{45} (1/min)	0.0114 (24)	0.0058–0.017
k_{54} (1/min)	0.00356 (17)	0.0024–0.0047
k_{61} (1/min)	0.00478 (11)	0.0037–0.0058
F_{abs}	0.299 (12)	0.23–0.37
k_{87} (1/min)	0.00954 (8)	0.0081–0.011
k_{70} (1/min)	0.0446 (20)	0.027–0.062
V_{max} (mg/kg/min)	0.547 (19)	0.34–0.76
k_m (mg/kg)	39.1 (28)	17–61
R^2	0.974	–
AIC	1,065	–

See legend of Fig. 3 for explanation of the parameters. *AIC* Akaike's information criterion [1]

systemic circulation by reducing the first-pass metabolism by intestinal and liver CD and could increase, and prolong, plasma concentrations of cytidine analogues by decreasing their systemic metabolism by CD [5, 24, 29, 30]. Achieving and sustaining adequate THU concentrations is essential for the successful development of THU as a modulator of cytidine drug metabolism. We recently characterized the p.o. bioavailability of THU in mice, which was approximately 20% [3, 32]. An improved bioavailability of THU could result in less frequent p.o. dosing and more effective inhibition of CD in vivo. taTHU was synthesized as a lipophilic prodrug of THU that might increase the p.o. bioavailability of THU by enabling direct transmembrane absorption and potentially bypassing capacity-limited intestinal transporters. The ribose triacetylation approach was previously validated to improve the p.o. bioavailability of 6-azauridine and uridine [2, 7, 20].

THU displayed a longer terminal half-life after administration of i.v. taTHU then after administration of i.v. THU, suggesting slow redistribution of taTHU from tissues into plasma, as reflected in compartment 5 in the compartmental model. The rate constant k_{54} corresponds to a half-life of 195 min, which is close to the long half-life estimated non-compartmentally. The multi-compartmental nature of taTHU disposition parallels the disposition of THU [3, 13, 21, 25], and the slower tissue to plasma redistribution of taTHU reflects the higher lipophilicity of taTHU relative to THU. Our data also suggest that after p.o. administration of taTHU, most of the taTHU is converted to THU before reaching the systemic circulation. The higher lipophilicity of taTHU likely results in prolonged or higher tissue exposure to THU after i.v. administration of taTHU, but not after p.o. administration of taTHU.

The hydrolysis of taTHU to THU appears to be very rapid. This is evidenced by: 1) the failure to detect taTHU in plasma samples; 2) the early (5 min) THU T_{max} observed after i.v. taTHU; and 3) the similar THU plasma concentration *versus* time profiles after p.o. THU and p.o. taTHU. Furthermore, the inability to model all individual hydrolysis steps between taTHU and THU, and the absence of acetyl analogues of THU in urine after p.o. administration of taTHU suggests rapid sequential hydrolysis of all 3 ester bonds once taTHU enters the catalytic site. These data also suggest that after p.o. administration, taTHU is essentially quantitatively metabolized to THU by first-pass metabolism. Similarly, after p.o. administration of triacetyl-6-azauridine to humans, high levels of 6-azauridine, but no triacetyl or diacetyl, and only minute levels of monoacetyl-6-azauridine were observed in serum [10].

The non-compartmental and compartmental pharmacokinetic analyses agree with respect to the fraction of p.o. administered taTHU that is absorbed and results in THU (approximately 30%) and the fraction of i.v. administered taTHU that is converted to THU (approximately 68%). Our data suggest that the addition of three acetyl moieties to THU increases availability of THU from 20 to 30%, which represents a 50% increase. Translation of this finding to the clinical situation requires recognition of the fact that p.o. THU appears to be less bioavailable in humans than in mice. The approximately 10–14% p.o. bioavailability of THU in humans [21, 25] might allow for a more profound improvement in THU delivery by the more lipophilic taTHU prodrug.

THU and cytidine analogues may compete with each other for absorption via gut transporters and/or carriers, which may result in decreased absorption of and exposure to THU, as observed after p.o. but not i.v co-administration of gemcitabine with THU (twofold decrease) [4]. It would be expected that such a competition would not occur with taTHU, which is more likely to be taken up by passive absorption than by carriers or transporters. Similarly, the related compound triacetyluridine does not require transporters for absorption [33]. Uridine p.o. bioavailability in mice is approximately 7% and could be improved sevenfold by administration of the triacetylated prodrug [2, 23]. Uridine p.o. bioavailability in humans [35] is approximately 7% as well, comparable to that of THU. Clinically, triacetyluridine resulted in a nearly tenfold increased p.o. availability of uridine, approaching complete bioavailability [20, 35]. Triacetyluridine (PN401, vistonuridine) is

currently being used as an antidote against 5-FU overdosing under an FDA orphan drug designation [36]. After p.o. dosing of [^{14}C]-triacetyl-6-azauridine to humans, the urinary excretion of deacetylated 6-aza-uridine and acetylated 6-azauridine was 80 and 17% of the dose, respectively [19]. The lower apparent p.o. absorption of taTHU in mice compared with the absorption of triacetyl-6-azauridine in humans suggests that p.o. bioavailability of taTHU in humans may well be higher than currently observed in mice.

Our data indicate that THU concentrations >1 μg/mL, which are known to inhibit CD [13, 28], are readily achieved in plasma for at least 2.5 h. However, because taTHU was not directly quantitated, we do not have information on its volume of distribution, which prevents us from inferences on the tissue exposure to taTHU or THU. Because taTHU is more lipophilic than THU, it is possible that THU is produced from taTHU in tissues and does not reappear in plasma. Consequently, the inhibition of CD in tissues may be underestimated by only considering plasma concentrations of THU. However, the data also indicate that this consideration is only relevant after i.v. administration of taTHU. Ultimate validation of the use of taTHU to inhibit cytidine drug catabolism is to perform studies of the combination of taTHU and a cytidine drug. However, we feel the pharmacology of THU is sufficiently characterized that the plasma exposure to THU after taTHU administration may be used as a marker of the inhibition of CD after taTHU administration.

THU has a very good safety profile in a variety of animal species, as reviewed [3]. A difference in the safety profile of taTHU would likely be due to: (1) different distribution characteristics or (2) the nature of taTHU and/or the intermediates. The most obvious difference between THU and taTHU is the presence of acetyl moieties which, during hydrolysis, are released as acetic acid. At sufficiently high doses, this might result in local acidosis. With the currently applied human THU dose of 300 mg/m^2 [6, 31], the equivalent taTHU dose of 450 mg/m^2, in an average person of 1.76 m^2, with complete absorption 47%, assuming complete hydrolysis, could theoretically result in the generation of approximately 809 mg or 13 mmol of acetic acid. The buffer capacity of human blood is 38.5 mEq/L [14]. Assuming a blood volume of 5 L, an immediate generation of 13 mmol acetic acid would change the pH of blood by 0.06 pH units, which is negligible. Depending on the rate of production and how locally the acetic acid is produced (e.g. in the liver where a large metabolic capacity resides), this may yet have relevance to the safety profile. However, triacetyluridine and its metabolites were reported to be non-toxic in animal toxicology tests [11].

In conclusion, the data presented here suggest that taTHU modestly improves the delivery of THU after p.o. dosing relative to an equimolar dose of THU. Administration of taTHU appears to result in a more prolonged exposure of THU, but the relevance of this extended profile is unclear. Clinical studies will be required to define the real value of p.o. taTHU towards efficient delivery of THU in humans.

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Conflict of interest statement None.

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