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SHORT COMMUNICATION

Concentrations of the DNA methyltransferase inhibitor 5-fluoro-2′-deoxycytidine (FdCyd) and its cytotoxic metabolites in plasma of patients treated with FdCyd and tetrahydrouridine (THU)

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Abstract
Purpose Although the DNA methyltransferase inhibitor 5-fluoro-2′-deoxycytidine (FdCyd), is being evaluated clinically, it must be combined with the cytidine deaminase inhibitor tetrahydrouridine (THU) to prevent rapid metabolism of FdCyd to the pharmacologically active, yet unwanted, metabolites 5-fluoro-2′-deoxyuridine (FdUrd), 5-fluorouracil (FU), and 5-fluorouridine (FUrd). We assessed plasma concentrations of FdCyd and metabolites in patients receiving FdCyd and THU.
Methods We validated an LC-MS/MS assay, developed for a preclinical study, to quantitate FdCyd and metabolites in human plasma. Patients were treated with five daily, 3-h infusions of FdCyd at doses of 5–80 mg/m^2 with 350 mg/m^2 THU. Plasma was obtained during, and before the end of infusions on days 1 and 5.
Results The lower limits of quantitation for FU, FdUrd, FUrd, FC and FdCyd were 1, 1.5, 10, 3, and 10 ng/ml, respectively. Plasma FdCyd increased with dose, from 19–96 ng/ml at 5 mg/m^2 to 1,600–1,728 ng/ml at 80 mg/m^2. FdUrd was undetectable in patients treated with FdCyd doses <20 mg/m^2, and increased from 2.3 ng/ml at 20 mg/m^2 to 3.5–5.7 ng/ml at 80 mg/m^2. FU increased from 1.2–5.5 ng/ml at 5 mg/m^2 to 6.0–12 ng/ml at 80 mg/m^2.
Conclusions By co-administering FdCyd with THU, FdCyd plasma concentrations were achieved that are known to inhibit DNA methylation in vitro. The accompanying plasma FU and FdUrd concentrations are <10% those

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observed after therapeutic infusions of FU or FdUrd, while FdCyd levels are well above those required to inhibit methylation in vitro. Therefore, inhibition of DNA methylation with FdCyd and THU appears feasible.

Keywords Metabolism · Pyrimidine · DNA methylation inhibitor · Phase I · Tetrahydrouridine

Introduction

Epigenetic changes, such as aberrant promoter hypermethylation associated with inappropriate gene silencing, affect virtually every step in tumor progression [8]. 5-azacytidine and 5-aza-2′-deoxycytidine, both recently licensed for human use, target DNA methyltransferases, the enzymes responsible for promoter hypermethylation [8, 15, 16]. However, the poor aqueous stability of these tri-aza compounds has limited their administration to rapid parenteral dosing.

The nucleoside analogue 5-fluoro-2′-deoxycytidine (FdCyd, NSC 48006) is a DNA methylation inhibitor that is stable in aqueous solutions. Unfortunately, in vivo, FdCyd is rapidly converted by cytidine deaminase (CD; EC 3.5.4.5) [5] to 5-fluoro-2′-deoxyuridine (FdUrd), followed by metabolism to 5-fluorouracil (FU) and 5-fluorouridine (Furd; Fig. 1). These metabolites do not contribute to the hypomethylating effect of FdCyd, and have cytotoxic effects that could be considered side-effects if only reversal of aberrant promotor hypermethylation is intended [3, 18]. 3,4,5,6-tetrahydrouridine (THU) is a potent CD inhibitor that has previously been used pre-clinically and clinically to prevent metabolism of cytidine analogues [1, 2, 4, 6, 11, 12, 14, 15, 19]. Co-administration of FdCyd with THU could delay and diminish conversion of FdCyd to its metabolites and thereby result in less cytotoxic side-effects and more efficient hypomethylation [3, 18]. The combination of i.v. FdCyd and i.v. THU is currently being investigated in a phase I study [15].

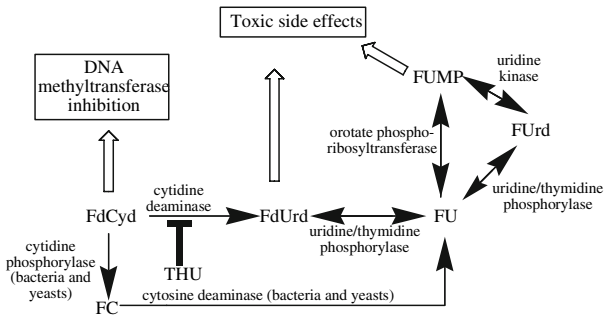

Fig. 1 Metabolism of 5-fluoro-2′-deoxycytidine (FdCyd). Metabolites are 5-fluoro-2′-deoxyuridine (FdUrd), 5-fluorocytosine (FC), 5-fluorouracil (FU), 5-fluorouridine (FUrd), and 5-fluorouridine monophosphate (FUMP). Tetrahydrouridine (THU) blocks the conversion of FdCyd to FdUrd by cytidine deaminase. *Solid arrows* represent metabolic pathways, *open arrows* represent drug effects

We recently developed an assay for the quantitation of FdCyd, FdUrd, FU, and FUrd in mouse plasma and used it to describe the p.o. bioavailability and metabolism of FdCyd in mice treated with FdCyd, with and without THU [1]. We realized that this assay, after cross-validation to human plasma, would be excellently suited to confirm that FdCyd co-administered with THU could achieve relevant FdCyd plasma concentrations and to assess the exposure of the phase I patients to the unwanted metabolites of FdCyd. The objective of the current study was to quantitate the concentrations of FdCyd, FdUrd, FU, and FUrd in plasma samples of the patients participating in the phase I study of FdCyd and THU [15].

Materials and methods

Chemicals and reagents

The FdCyd (25 mg/5 ml water) for injection was manufactured for City of Hope by University of Iowa Pharmaceutical Service Division and administered under IND #54,223. Nonsterile tetrahydrouridine (700 mg) was manufactured by City of Hope and provided for reconstitution in water for injection and sterile filtration at the time of administration under IND #54,223. FdCyd, FdUrd, FU, FUrd, and THU for analytical purposes were provided by the Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD). 2-pyrimidinone and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile, ethyl acetate, glacial acetic acid, sodium phosphate dibasic, and potassium phosphate monobasic were purchased from Fisher Chemicals (Fair Lawn, NJ). All reagents were of analytical grade. Water was purified using a Q-gard® 1 Gradient Milli-Q system (18.2 MΩ cm, Millipore, Billerica, MA). Human plasma for preparation of the calibration samples was produced by centrifuging whole blood (Central Blood Bank, Pittsburgh, PA) for 20 min at 2,000 × *g* at room temperature.

Patients

Patients were enrolled in a phase I study of FdCyd and THU [15]. Before enrollment, all subjects signed an informed consent document approved by the Institutional Review Board of the City of Hope National Medical Center. FdCyd was administered i.v. at 5, 10, 20, 40, 60, and 80 mg/m^2 over 3 h, daily for 5 days, the first week of every 3 weeks. THU was dosed at 350 mg/m^2 (as described before in combination with cytosine arabinoside [10]), with 20% administered as an i.v. bolus loading dose immediately

before, and 80% infused concomitantly with FdCyd. FdCyd and THU for the 3-h infusions were diluted in 250 ml of water for injection.

Plasma sampling

Heparinized blood samples were obtained on days 1 or 5 from patients treated at doses of 5 ($N = 6$), 10 ($N = 3$), 20 ($N = 1$), 40 ($N = 6$), and 80 ($N = 4$) mg/m^2. Samples were obtained between 1.5 h after the start of the infusion and just before the end of the infusion (median 2.7 h). Blood samples were placed on ice immediately and centrifuged at 600 × *g* for 10 min at 4°C to obtain plasma. The plasma was transferred to a tube containing THU (to prevent ex vivo FdCyd deamination) and stored at −20°C until analysis. FdCyd, FdUrd, FU, FUrd, and 5-fluorocytosine (FC) in plasma were quantitated with a hydrophilic interaction chromatography (HILIC) [17] HPLC-MS/MS assay (see below). Plasma concentrations of FdCyd, FdUrd, and FU on days 1 and 5 were compared with a paired Student's *t* test.

Cross-validation of the assay for quantitation of FdCyd and its metabolites

The assay as previously described for the quantitative analysis of FdCyd, FdUrd, FU, FUrd, and FC in mouse plasma [1] was cross-validated in human plasma. Precision and accuracy were assessed at 4 concentrations ($N = 6$) spanning the dynamic calibration ranges, and extraction recovery was assessed at 1/3 the concentration of the upper limit of quantitation. Recovery was defined as the absolute response of an extracted sample relative to the response of an extracted blank with the compounds added after reconstitution. Dynamic calibration ranges were: FdCyd, 10–3,000 ng/ml; FdUrd, 1.5–1,500 ng/ml; FU, 1–300 ng/ml; FUrd, 10–3,000 ng/ml; and FC, 10–3,000 ng/ml.

Results

Assay performance and retention times for human plasma samples, are displayed in Table 1. Accuracy was between 80 and 115%, and precision was within 19% for all compounds.

Plasma concentrations of FdCyd during the infusion are displayed in Fig. 2, while the concentrations of FdCyd and its metabolites are presented in Table 2. FdCyd concentrations increased linearly with the dose of FdCyd. The concentrations of the deaminated metabolites FdUrd and FU also increased with dose, although less steeply. FUrd and FC were each observed in only one sample. The FdCyd, FdUrd, and FU concentrations on days 1 and 5 did not differ significantly. Pre-treatment samples did not contain FdCyd or any of the metabolites.

Discussion

The objective of the current study was to quantitate the concentrations of FdCyd, FdUrd, FU, Furd, and FC in plasma samples of the patients participating in a phase I study of

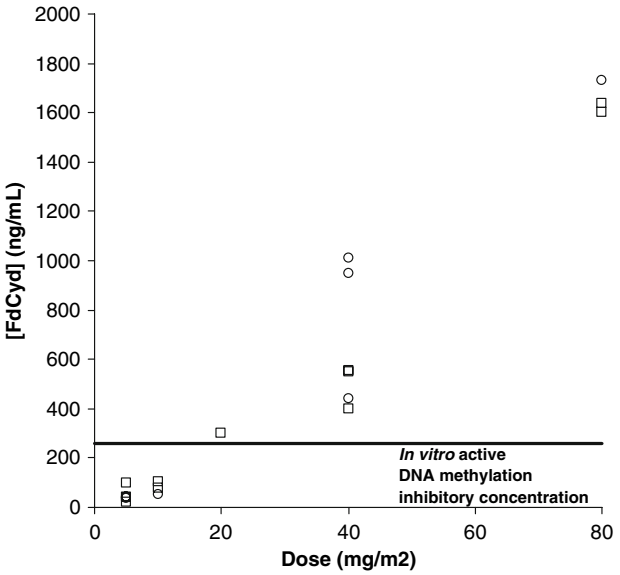

Fig. 2 End of infusion plasma concentrations of FdCyd achieved after i.v. administration of various doses of FdCyd co-administered with 350 mg/m^2 THU on day 1 (*open square*) or day 5 (*open circle*) in patients participating in the phase I trial of FdCyd and THU

Table 1 Assay parameters for FdCyd and its metabolites

Compound	Rt (min)	Ionization mode	MRM (*m/z*)	MRM window (min)	Accuracy (%)	Precision (%)	Recovery (%)
FdCyd	25.6	+	130–113	22–49	88–110	4.2–13	5.4
FdUrd	9.4	–	245–155	0–12	80–115	3.2–16	60
FU	8.8	–	129–41.5	0–12	97–108	5.5–19	62
Furd	18.1	–	261–171	16.5–22	94–106	3.5–10	25
FC	24.3	+	130–113	22–49	88–103	2.9–8.7	13
IS	12.8	+	97–79	12–16.5	–	–	–

Rt retention time

Table 2 Plasma concentrations of FdCyd and its metabolites during the infusion

Subject	Dose (mg/m^2)	Day	Time after start infusion (h)	FdCyd (ng/ml)	FdUrd (ng/ml)	FU (ng/ml)
1	5	1	2.9	96	ND	5.5
1	5	5	3.0	36	ND	4.0
2	5	1	2.7	19	ND	2.6
2	5	5	2.7	41	ND	1.4
3	5	1	3.0	40	ND	1.2
3	5	5	1.8	40	ND	1.4
4	10	1	NA	77	ND	ND
4	10	5	2.5	49	ND	ND
5	10	1	2.5	103	ND	1.3
6[a]	20	1	2.8	300	2.3	4.7
7[b]	40	1	2.5	554	4.2	3.0
7	40	5	1.5	1010	5.0	7.2
8	40	1	2.9	548	1.7	4.9
8	40	5	2.6	441	2.6	5.5
9	40	1	2.9	399	2.1	2.7
9	40	5	2.8	950	ND	5.5
10	80	1	2.5	1600	3.5	12
10	80	5	2.5	1728	3.6	8.1
11	80	1	2.7	1640	5.7	6.0

NA not available, *ND* not detected

[a] In this sample, FUrd was detected and determined to be present at a concentration of 16 ng/ml

[b] In this sample, FC was detected and determined to be present at a concentration of 13 ng/ml

FdCyd and THU [15]. Our previously published assay for the quantitative analysis of FdCyd and its metabolites in mouse plasma [1] had a similar performance in human plasma.

Because of the short half-lives of the analytes, at 1.5 h after start of infusion, the FdCyd and metabolite concentrations were expected to have reached steady-state. Therefore, samples obtained before the end of infusion and samples obtained during the infusion can directly be compared.

As expected, the concentrations of FdCyd and its metabolites increased with the dose of FdCyd administered. At doses as low as 20 mg/m^2, the observed FdCyd concentrations exceeded 260 ng/ml, which has been shown to inhibit in vitro DNA methylation in MCF-7 cells (24 h incubation) [9]. Although the observed FdCyd plasma concentrations are maximum concentrations that decrease with time after completion of the FdCyd infusion, the THU co-dosed in the current study is expected to prolong intra-cellular exposure to FdCyd by blocking cellular CD. Obviously, an in vivo pharmacodynamic outcome of effectiveness, or a biomarker for such effectiveness, such as DNA demethylation or re-expression of genes, will be decisive in establishing the minimal effective dose. However, the pharmacokinetic data are encouraging and suggest that the intended biological effect may occur at an FdCyd dose level that is below the maximum tolerated dose, which has not yet been reached.

We detected FdUrd and FU in most of the samples, and detected FUrd and FC each in one sample. We did not expect to see FUrd in many samples because it is the third metabolite in the enzymatic conversions downstream from FdCyd. In addition, the first enzymatic step, from FdCyd to FdUrd, is strongly inhibited by the presence of THU, as was shown in earlier pre-clinical work [1]. The presence of FC in one of the samples was unexpected. FC is usually seen only after p.o. dosing of FdCyd, which exposes the parent drug to the gut bacteria that, unlike mammalian cells, are capable of converting FdCyd to FC [1]. At the highest FdCyd dose administered, the maximum FdUrd and FU concentrations observed were 5.7 and 12 ng/ml, respectively. Our earlier pre-clinical studies in mice [1] showed that after a combined i.v. bolus of FdCyd and THU, the highest metabolite concentrations (FdUrd and FU) were observed at the earliest sample and then exhibited a slow decline, which implied that the clearance of these FdCyd metabolites was more rapid than their formation. Consequently, the metabolite concentrations observed in the current clinical study are not expected to increase after the end of infusion, but to decrease slowly with time. In mice, the addition of THU increased the half-life of FdCyd 5-fold, and reduced the C_{max} of its metabolites 14-fold [1]. Because the steady-state concentration of an infused drug is dependent on its clearance, this means that if FdCyd were given without THU, the dose required to reach similar plasma concentrations would be five times as high. The profile in

Fig. 2 then suggests that at a dose of 80 mg/m^2 FdCyd, without THU, active plasma concentrations of FdCyd would be achievable. However, the absence of THU would allow for toxic concentrations of FU and FdUrd to be generated. The plasma concentration versus time profile of FdUrd and FU after i.v. administration of FdCyd and THU to mice resembles that of a continuous infusion of FdUrd or FU, respectively [1]. Clinically, FdUrd may be administered directly for its cytotoxic effects as an extended infusion at a dose of 30 mg/kg/8 h, which results in plasma concentrations of both FdUrd and FU of approximately 200 ng/ml [7]. Similarly, continuous FU infusions of 400 mg/m^2/day or 30 mg/kg/8 h result in FU plasma concentrations of approximately 100 and 900 ng/ml, respectively [7, 13]. Even at the highest FdCyd dose level in our study, the observed maximum plasma concentrations of FdUrd ($\leq$5.7 ng/ml) and FU (12 ng/ml) are more than 8-fold lower then the plasma concentrations observed when FdUrd or FU are administered by continuous infusion as cytotoxic therapy. The relevance of FdUrd and FU to the toxicity profile of FdCyd coadministered with THU under the current clinical schedule remains to be determined and is one of the objectives of the ongoing clinical phase I study.

We obtained plasma samples from patients on days 1 and 5 of treatment. Although our study was not primarily powered to detect a significant difference in pharmacokinetics between days 1 and 5, the absence of any difference, e.g. due to accumulation, is in agreement with the short half-lives of the compounds involved.

Accrual to the phase I study from which the samples presented here were obtained is ongoing. Based on pre-clinical data indicating that a duration of exposure to FdCyd of greater than 1 week may be important, the protocol has been amended such that treatment is now being given daily five times for 2 weeks out of a 4-week cycle. Although we are continuing to collect plasma samples from patients so that we can characterize the plasma concentration versus time profile of FdCyd and its cytotoxic metabolites more completely, the data presented here provide encouraging evidence that FdCyd levels required to inhibit DNA methylation are achievable. Furthermore, plasma concentrations of the toxic metabolites of FdCyd (FdUrd and FU) are well below the levels seen when these compounds are given therapeutically.

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