Journal of Chromatography B 1084 (2018) 4–13

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

Simultaneous determination of gemcitabine prodrug, gemcitabine and its major metabolite 2′, 2′-difluorodeoxyuridine in rat plasma by UFLC-MS/MS

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ARTICLE INFO

Keywords:
Gemcitabine
Prodrug
Metabolic activation
UFLC-MS/MS
Nucleoside

ABSTRACT

To improve bioavailability and provide resistance to deamination, an array of gemcitabine (dFdC) prodrugs carrying the acyl modifications has been successful in the optimization of pharmacokinetic properties of dFdC, but the reports about 4-*N*-carbobenzoxy-dFdC (Cbz-dFdC), a dFdC prodrug bearing alkyloxycarbonyl modification, are relatively rare. Notably, *in vivo* enzymatic hydrolysis was an absolutely essential factor for the activation of these prodrugs, which is correlated with the anti-tumor activity. Therefore, detailed metabolism studies of Cbz-dFdC should be carried out for a more authentic pharmacodynamic evaluation. In order to detect the pharmacokinetic characteristics of Cbz-dFdC, a selective, sensitive and accurate method for the simultaneous determination of Cbz-dFdC, along with dFdC and its major metabolite dFdU in rat plasma was developed and validated using UFLC–MS/MS techniques. Column was at 40 °C for separation using an eluent with acetonitrile and 0.1% formic acid, 1 mM ammonium formate at a flow rate of 0.2 mL/min. Detection was performed using ESI source in positive ion selected reaction monitoring mode by monitoring the following ion transitions *m/z* 398.1 → 202.2 (Cbz-dFdC), *m/z* 264.1 → 112.0 (dFdC), *m/z* 265.3 → 113.2 (dFdU) and *m/z* 246.1 → 112.0 (IS). Analytes were extracted by simple precipitation with acetonitrile containing internal standards followed by liquid-liquid extraction with ethyl acetate. The calibration curves of Cbz-dFdC, dFdC and dFdU were linear in the concentration range of 2 to 500 ng/mL, 2 to 500 ng/mL and 40 to 10,000 ng/mL, respectively. The assay ranges selected for the three analytes were appropriate and minimized the need for reanalysis. All the validation data, such as intra- and inter-day precision, accuracy, selectivity and stability, were within the required limits. In conclusion, the sensitive analytical assay was selective and accurate for the determination of rat plasma concentrations of Cbz-dFdC, dFdC and dFdU from a single LC–MS/MS analysis and well-suited to support pharmacokinetic studies.

1. Introduction

Gemcitabine (dFdC, 2′, 2′-difluorodeoxycytidine, Gemzar®), a marketed deoxycytidine analog bearing a *gem*-difluoromethylene in the deoxyribose moiety (Fig. 1), demonstrates significant *in vitro* and *in vivo* anticancer activity though the inhibition of DNA synthesis [1,2]. It is considered as the golden standard of advanced pancreatic cancer treatment, and also used for the treatment of various types of cancers such as metastatic breast cancer, non-small cell lung cancer and bladder cancer [3–6]. After intravenous injection, dFdC undergoes two main metabolic pathways. The intracellular phosphorylation of dFdC could lead to the formation of active diphosphate and triphosphate metabolites, resulting in the antitumor activity [7–10]. Notably, an undesired deamination of dFdC widely and rapidly occurs in plasma and liver by cytidine deaminase (CDA), affording the inactive metabolite 2′, 2′-difluorodeoxyuridine (dFdU, Fig. 1) and resulting in a steeply elimination of dFdC [9–11].

Compared to the high-dose dFdC treatment, lower doses administrated over longer durations have been proved to be effective for the hematotoxicity and gastrointestinal toxicity reducing and the improvement of survival and quality of life in clinical studies [12,13]. In addition, oral administration was considered to be superior to the complex intravenous treatment schedule of dFdC used in clinical. Therefore, an array of dFdC prodrugs bearing acyl modifications was reported to provide low but long-lasting oral doses of dFdC and resistance to deamination. A close structural analog LY2334737 (phase II clinical trial, Fig. 1) carrying 4-*N*-(2-propyl)pentanoyl was designed and developed to achieve prolonged systemic exposure of dFdC, taking

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https://doi.org/10.1016/j.jchromb.2018.03.025
Received 21 December 2017; Received in revised form 6 March 2018; Accepted 12 March 2018
Available online 14 March 2018
1570-0232/

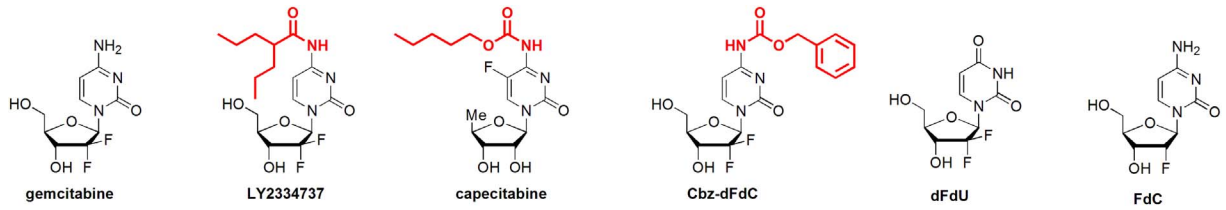

Fig. 1. Structures of gemcitabine and the related prodrugs.

advance of the high CDA stability of the amide moiety [14–16]. Similarly, other dFdC prodrugs, such as 4-*N*-stearoyl dFdC and 4-*N*-squalenoyl dFdC, also possessed hydrolysable amide modification, improving the bioavailability and metabolic stability of dFdC [17,18]. In sum, the acyl modifications have been successful in the improvement of pharmacokinetic properties of dFdC, which prompt us to carry out the further exploration for the diversity of the prodrug structures [19].

Capecitabine (marketed, Fig. 1) was a prodrug of 5-fluorouracil (5-FU), bearing (pentyloxy)carbonyl to the amino of cytosine, which undergoes the metabolic activation by carboxylesterase (CES) and CDA successively [20–22]. Furthermore, different influences on the activities between acyl and alkyloxycarbonyl modification of nucleoside prodrugs were recently recognized in our group [23], so we are curious to confirm whether the alkyloxycarbonyl modification could be applied in the optimization of dFdC and choose the Cbz-dFdC for further research. Strikingly, no pharmacokinetic results of any alkyloxycarbonyl dFdC have been reported, encouraging us to pour more attention to the cellular and *in vivo* pharmacokinetic studies [25]. In 2014, Weiss reported that 4-*N*-carbobenzoxy-dFdC (Cbz-dFdC, Fig. 1) had little effect on the growth of BxPC-3 cell line [14], which might attribute to the lower levels of esterase expression. Similarly, LY2334737 produced no negative effect on cell viability in HCT-116 and HEK293, resulting from the deficiency of CES2 expression [24]. Therefore, to precisely profile the metabolism details of Cbz-dFdC in rat, a sensitive and reliable UFLC-MS/MS method was established and successfully verified for the simultaneous quantification of Cbz-dFdC, together with the corresponding metabolites dFdC and dFdU.

2. Experimental

2.1. Materials

Analytical grade reagents such as formic acid were supplied by Acros (Belgium, EU) with 98% purity. Acetonitrile was from Merck LiChrosolv (Darmstadt, Germany). Ammonium formate was purchased from Adamas-beta Company (Shanghai China) with 99% purity. HPLC grade ethyl acetate was purchased from Alligator Reagent (Tianjin, China) with 99.8% purity. MilliQ water purification system (Millipore, Amsterdam, The Netherlands) was used to obtain HPLC grade water purified at 18.2 MΩ, which was directly into glass containers using a 0.22 μm millipore filter.

Reference compound dFdC was provided by Energy Chemical (Shanghai, China). Cbz-dFdC was synthesized in our own lab with 98% purity and verified by ^{1}H NMR. 2′, 2′-difluorodeoxyuridine (dFdU) were supplied by TRC (Toronto, Canada) with 98% minimum purity. Internal standard 2′-fluorodeoxypyrimidine (FdC, Fig. 1) was provided by Aladdin Industrial Corporation (Shanghai, China) with a purity of 98%.

2.2. Equipment

An Eppendorf AG 22331 centrifuge (Hamburg, Germany) was used for blood centrifugation in 1.5 mL EP tube. Thermo fisher electron LED GmbHD-37,520 Biofuge (Osterode, Germany) was used for plasma extraction. Scientific Industries Vortex Genie 2 (USA) was used for mixing. Termovap sample concentrator used for drying sample was supplied by Qi Qian (Shanghai, China).

2.3. Chromatography

Phenomenex Synergi™ Fusion-RP (150 × 2 mm, 4 μm, 80 Å) HPLC Column was used. The temperature of column and sample injector was 40 °C and 4 °C respectively. The column protection was 2 μm and also supplied by Phenomenex. Eluent A was 1 mM ammonium formate and 0.1% formic acid in water and eluent B was pure acetonitrile. The gradient was as follows: 10% B for 0–0.5 min, ramped up to 82% B for 0.5–3.5 min following up to 90% B for 3.5–6.5 min and held 90% B for 6.5–8.0 min to wash off the low-polarity impurities in the column, after which the flow goes back to the initial gradient using approximately 0.5 min and held for 8.5-11 min. All of transitions were linear, and the acquisition time of a single analysis was about 11 min. The flow rate was 0.2 mL/min from the beginning to the end.

The Shimadzu UFLC system (Kyushu Island, Japan) consisted of an on-line vacuum degasser, pulse damper, gradient pumps, an auto-sampler and a column thermostat between column and MS source. The injection volume was 5 μL using a 20 μL loop on a CTC HTS PAL system (Carrboro, NC, USA). The auto-sampler used a mixture of 1:1 water and methanol (*v*/v) to rinse needle.

2.4. Mass conditions

The MS/MS spectrometry detector was AB Sciex API 4000 + ™ LC-MS/MS (USA), a triple quadrupole mass spectrometer with a Turbo Ion Spray source. And the software Analyst v.1.4.1 was used to acquire and process data. The parent and the daughter ion of internal standard and each analyte were detected by full scan and product ion spectra through infusing directly. The positive multi reaction monitoring (MRM) mode was developed to quantify Cbz-dFdC, dFdC, dFdU and IS, whose transition pairs (molecular ion and major fragment ion) was determined to *m/z* 398.1/202.2, *m/z* 264.1/112.0, *m/z* 265.3/113.2 and *m/z* 246.1/112.0, respectively. The optimized spectrometer conditions were as follows: 10/min for collision activated dissociation gas flow (CAD), 30/min for curtain gas flow, 500 °C for probe temperature. The ion spray voltage of the MS/MS was set to 5500 V. Ion Source Gas 1 and Ion Source Gas 2 was set to 50/min and 55/min, respectively. And the CXP and EP was 12, 10, respectively. The dwell times of the internal standard and three analytes were 100 msec. The corresponding collision energy and the declustering potential are 24, 25, 23, 17 (CE, eV) and 70, 60, 55, 55 (DP) for Cbz-dFdC, dFdC, dFdU and FdC, respectively.

2.5. Standard solutions

Independent standard and QC stock solutions of Cbz-dFdC, dFdC and dFdU were made at 1.0 mg/mL. All of them were dissolved in acetonitrile, and stored at 2–8 °C. The internal standard was also made into 1 mg/mL, stored in the similar environment. The working solution of internal standard was made from diluting 20 μL corresponding stock solution with pure acetonitrile, eventually forming concentration of 200 ng/mL. A working solution of Cbz-dFdC, dFdC/dFdU mixture was using a serial dilution procedure at 5000/100,000, 2000/40,000, 1000/20,000, 500/10,000, 200/4000, 100/2000, 50/1000, 20/400 and 10/200 ng/mL to make calibration standards in treated plasma. 5 μL calibration solution and 45 μL blank plasma were mixed as standard curves in 1.5 mL EP tube. Three concentrations of rat plasma (20, 200 and