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Sensitive analysis and pharmacokinetic study of a novel gemcitabine carbamate prodrug and its active metabolite gemcitabine in rats using LC-ESI-MS/MS

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Abstract

FY363 is a new chemical entity of gemcitabine analog, which has been shown to have a significant inhibitory effect on cell proliferation in a variety of tumor cell lines in vitro. As a carbamate derivative, FY363 would be converted to the active metabolite gemcitabine through enzyme action in vivo. In order to clarify the exposure of FY363 prototype and its metabolite gemcitabine in vivo after administration of FY363, a sensitive and specific liquid chromatography tandem mass spectrometry (LC-MS/MS) was developed and validated to simultaneously determine FY363 and gemcitabine in rat plasma after liquid-liquid extraction with ethyl acetate. Chromatographic separation was achieved on a highly stable polar column of Synergi 4u Polar-RP 80A (4μm, 4.6 × 250 mm) which has a unique ether - phenyl bonded phase. Gradient elution was accomplished with mobile phase system consisting of 5mM ammonium formate buffer containing 0.1% formic acid and mixed organic solvents containing methanol-acetonitrile (3:2, v/v). Multiple reaction monitoring transitions were performed on triple quadrupole mass spectrometric detection in positive-ion mode with an electrospray ionization source. The calibration curves showed good linearity ($r > 0.99$) over the established concentration range of 1.0 - 1000 ng/mL both for FY363 and gemcitabine. The assay was validated to be selective, robust and reproducible. This well validated method was successfully applied to demonstrate the pharmacokinetic behavior and the metabolic transformation of FY363 in rats. Results revealed that about 20% of FY363 were converted into its active metabolite gemcitabine in rats by comparing the exposure of gemcitabine after the FY363 administration with that after direct gemcitabine administration at equimolar dose.

Key words: FY363; Gemcitabine; LC-MS/MS; Rat Plasma; Pharmacokinetics;

1. Introduction

Gemcitabine is one of the most widely used pyrimidine analogues, with a well-established role as a first- and second-line treatment of several types of tumors, such as pancreas cancer, breast cancer, bladder cancer and non-small cell lung cancer [1-4]. Gemcitabine is hydrophilic and must be transported into cells by specialized nucleoside transporters such as SLC29A1 SLC28A1, and SLC28A3 [5, 6]. Once in cell, gemcitabine undergoes a series of phosphorylations into its monophosphate, diphosphate, and triphosphate derivatives, which ultimately block DNA synthesis [6-8].

Although gemcitabine has shown efficacy in many clinical situations, the drug is also associated with side effects such as flu-like symptoms, diarrhea, weakness, mouth ulcers and shortness of breath. Furthermore, gemcitabine is rapidly deaminated by cytidine deaminase (CDA), prevalent in plasma and liver, resulting in 2',2'-difluorodeoxyuridine (dFdU), which is noncytotoxic [9, 10]. This rapid metabolism of gemcitabine explains its poor bioavailability and short plasma half-life (32-84 min for short infusions in humans) [11-16]. In addition, one of the major difficulties in cancer therapy is that tumors acquire resistance over time. For gemcitabine, altered transport over the cell membrane, such as a decreased expression of nucleoside transporters, is a mechanism of resistance to gemcitabine by blocking its uptake to cancer cells [17]. Therefore, in order to improve safety and tolerability, overcome high metabolic bioevasion, provide enhanced transport and maximize the antitumor benefits, new forms of gemcitabine by chemical modification is widely reported [18-22].

Drug modification has already been used with other anticancer drugs to overcome some disadvantages of the parent molecule. For example, paclitaxel has been covalently attached to an acyl chain to obtain a lipophilic prodrug of paclitaxel to increase its encapsulation in lipid emulsion [23]. Cytosine arabinoside has been modified by grafting fatty acid chains at the 5'-position of the nucleoside to promote its uptake and to prolong its retention in cells [24]. Among various chemical modifications of gemcitabine, 4-(N)-position of the molecular (Fig. 1a) is a hot-spot

modification site. For example, LY2334737 (Fig.1b) is an oral gemcitabine prodrug in which gemcitabine is linked to valproic acid via an amide bond at 4-(N)-position, enabling it to block deamination by CDA [25]. LY2334737 is orally absorbed intact and is hydrolyzed by slow systemic cleavage resulting in prolonged gemcitabine exposure [18]. LY2334737 is a clinically effective amide prodrug of gemcitabine with good development prospects [18, 26-28]. Another example is CP-4125. To facilitate accumulation, an elaidic fatty acid group was acylated on the 4-amino group in CP-4125 to reduce deamination. Accumulation of dFdCTP (2',2'-difluorodeoxycytidine triphosphate, gemcitabine's active form) pools after CP-4125 exposure increased for at least 20 hr [29]. In addition to acylation modifications, alkoxycarbonyl modifications have also been successfully applied to nucleoside drugs. For example, capecitabine (Fig.1c) is a carbamate derivative of fluoropyrimidine deoxyribonucleoside with a pentyloxycarbonyl group linked to the amino group of cytosine.

FY363, 4-(Butyloxycarbonylamino)-1-[(2R,4R,5R)-3,3-difluoro-4-hydroxy-5-(hydroxymethyl)oxolan-2-yl]-1,2-dihydropyrimidin-2-one, is a novel chemical entity of gemcitabine analog. It was proposed in the approved U.S. patent (US 20140134160A1) to be injected or orally administered. FY363 (Fig. 1d) is a carbamate prodrug in which gemcitabine is linked to a butyloxycarbonyl group through the amino group at 4-(N)-position. In our study, FY363 has demonstrated superior antitumor activity than the clinical trial drug LY2334737 in a variety of tumor cells including human lung cancer, breast cancer and kidney liver (see Supplementary data). Therefore, FY363 is also expected to be a potential new strategy for gemcitabine. In humans, LY2334737 is cleaved systemically to gemcitabine by carboxylesterase (CES) *in vivo* [28, 30]. Similar metabolic activation by CES also occurred in capecitabine [31, 32]. Thus, a gradual release of gemcitabine following cleavage of the carbamate bond would be expected for FY363. Therefore, to precisely profile the metabolism details of FY363 in vivo, there is a need to develop an analytical platform for simultaneous quantitative of FY363 and gemcitabine in biological matrix.

In this manuscript, we developed and validated a sensitive and specific liquid chromatography tandem mass spectrometry (LC-MS/MS) to simultaneously determine FY363 and gemcitabine in rat plasma using liquid-liquid extraction and gradient elution. This novel analysis strategy was successfully applied to a pharmacokinetic study of FY363 in rats. At the same time, in order to understand the metabolic fraction of FY363 converted to gemcitabine in vivo, an equimolar dose of gemcitabine was administered alone. According to our literature searching, this is the first report of the pharmacokinetic behavior of FY363 in vivo, which would certainly provide an important basis for the development of FY363.

2. Material and methods

2.1 Chemicals and reagents

FY363 (purity > 99%) was supplied by FANG YUAN Pharmaceutical Co., Ltd (Changzhou, JiangSu, China), gemcitabine (purity > 99%) and saxagliptin(internal standard, IS) were purchased from Selleck (Houston, TX, USA). (2-hydroxypropyl) -β-cyclodextrin was purchased from Ourchem (Guangzhou, GuangDong, China). Methanol, acetonitrile, ethyl acetate, formic acid, ammonium formate and sodium dodecyl sulfonate (SDS) were analytical grade and purchased from Sigma–Aldrich (St. Louis, MO, USA). Ultrapure water was prepared by Milli-Q Ultrapure water purification system (Millipore, Bedford, USA) .

2.2 Chromatographic and Mass spectrometer conditions

FY363, gemcitabine and IS were separated on a 250 mm × 4.60 mm Synergi 4u POLAR-RP 80A column (Phenomenex, Torrance, CA, USA). The chromatography was performed at 40℃, under gradient conditions. Gradient mobile phase system consisting of 0.1% formic acid added with 5mM ammonium formate (A) and methanol-acetonitrile (3:2, v/v) (B) was applied at a flow rate of 1.2 mL/min. The post-column split ratio was 1 : 3. A typical injection of 5μL was sufficient to obtain required sensitivity. Run time of 13 min with a gradient elution: 0.0-2.0 min (10% B), 2.0-5.0 min (10-70%B), 5.0-8.0 min (70-90% B),8.0-9.5 min (90% B), 9.5-10.0 min (90-10% B) and 10.0-13.0 min (10% B) were used.

The mass spectrometer was a Sciex API-4000 (AB Sciex, Redwood City, CA, USA) equipped with a electrospray ionization (ESI) source, operated in positive ionization mode, using multiple-reaction monitoring (MRM). The operating source conditions were optimized as follows: the ionSpray voltage was set at 5500 V, the turbo spray temperature at 600°C, the ion source Gas 1 at 65 Arb, the ion source Gas 2 at 70 Arb, the curtain gas at 30 Arb and the Collision Gas at 10 Pa. The declustering potential and collision energy were 80 V and 31 V for FY363, 40 V and 26 V for gemcitabine and 50 V and 27 V for IS, respectively. The entrance potential and collision cell exit potential were 10 V and 12 V for all the analytes. Detection of target ions [M + H]+ was at m/z 364.0 for FY363, m/z 264.0 for gemcitabine and m/z 316.2 for saxagliptin (IS). The precursor-to-product ion transitions used were 364.0 → 212.1, 264.0 → 112.2 and 316.2 → 179.8 for FY363, gemcitabine, and IS, respectively. The data was acquired and evaluated using Analyst 1.5.1 (AB Sciex) software.

2.3 Preparation of standard solutions and calibration samples

5mg of FY363 and gemcitabine were respectively dissolved in 0.1ml DMSO and diluted with 0.9ml methanol to prepare stock solutions at a concentration of 5 mg/ml. The combined working solutions of analytes in the desired concentration range were prepared by appropriate dilution of standard stock solutions with methanol. All the solutions were stored at 2-8°C and were brought to room temperature before use. The calibration standard (CS) samples were prepared by spiking blank plasma with respective combined working solutions. The concentrations of mixed CS samples in plasma were 1, 3, 10, 30, 100, 300, 500, 1000 ng/mL both for FY363 and gemcitabine.

2.4 Sample preparation

The liquid-liquid extraction was used for sample preparation. Whole blood samples were centrifuged for 5 min at 8000 rpm to prepare plasma samples which were stored at -80 °C. 500μL ethyl acetate containing of 50ng/ml saxagliptin (IS) was added to 50μL of thawed plasma sample. After vortexing for 5 min and centrifuging

for 5min at 10000rpm, 450μL supernatant was collected and evaporated under a stream of nitrogen. The extract was then reconstituted in 100μL of methanol-water (9:1, v/v). The mixture was then centrifuged at 18000rpm for 10 min, and 5 μL supernatant was injected for analysis.

2.5 Analysis method validation

To assess the specificity, blank plasma samples, plasma samples spiked with single analyte, plasma samples spiked with mixed analyte and plasma samples after administration of drugs were detected. A eight-point linear calibration curve was constructed using a weighted (1/x) least squares linear regression by plotting the peak area ratios of analyte/IS versus nominated plasma concentrations over the range of 1.0 - 1000 ng/mL both for FY363 and gemcitabine. The lower limit of quantitation (LLOQ) is defined as the lowest concentration on the standard curve. LLOQ should meet the analytical requirements that S/N > 10 and the accuracy and precision were up to ± 20% relative error (RE, %) and relative standard deviation (RSD, %), respectively. Intra-day precision and accuracy were evaluated for six replicate samples at three concentration levels on the same day. Inter-day precision and accuracy were analyzed by the determination results in three consecutive days at three concentration levels. The accuracy was expressed as RE (%), and the precision as RSD (%). According to US Food and Drug Administration guidelines for bioanalytical method validation (US Food and Drug Administration, 2001)[33], both RE and RSD were expected to be within ± 15% to be acceptable.

Extraction recovery was calculated by comparing the peak areas of extraction samples and post-extraction spiked samples. Matrix effect was evaluated by comparing the peak areas of post-extraction spiked samples and pure standard solutions. Post-extraction spiked samples were prepared as follows: adding standard working solution into the residue after extraction of blank plasma to yield equivalent concentrations to corresponding extraction samples.

Six replicates at three concentration levels in plasma were used for stability validation under a variety of storage and handling conditions. Samples were subjected

to three freeze-thaw cycles, short-term frozen at -80℃ for 7 days and storing at room temperature for 6 h. Three freeze-thaw cycles mean freezing the plasma sample at -80℃ and then thawing at room temperature, thus repeating three times. Post-preparative stability was evaluated by reanalyzing post-extraction samples kept in the autosampler at 4℃ for 24 h.

2.6 Pharmacokinetic study in rats

Twelve Spague-Dawley rats (Male, 6-8 weeks old, 190-210g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). Animals were housed in an environment which controlled room temperature at 22 ± 2℃ and humidity at 55 ± 5 %. Animals received 12/12 h light/dark cycle. Prior to the experiment, rats were fed a standard diet for one week to adapt to the laboratory conditions. Animal welfare and experimental procedures weree strictly in accordance with the Guidelines of Animal Experiments of China Pharmaceutical University (Nanjing, China) and approved by the Animal Ethics Committee.

Dose solutions for intravenous administration were prepared by dissolving FY363 or gemcitabine in 50% (2-hydroxypropyl) -β-cyclodextrin aqueous solution. Rats were randomly divided into two groups, each group of six. One group was administered with FY363 and the other group was administered with gemcitabine at an equimolar dose of 50 μmol/kg by intravenous injection. Before administration, rats were fasted for 12 h. 150μL of blood samples were collected from the ophthalmic veins at 5min，15min，30min，1h，2h，4h，6h，8h，10h，12h and 24 h after administration using heparin tubes added with 7.5μL of 0.1 g/ml SDS aqueous solution to inactivate plasma carboxylesterase activity. Blood samples were subsequently processed as mentioned in section 2.4. The pharmacokinetic parameters were calculated by Phoenix WinNonlin 6.0 (Pharsight, Mountain View, CA).

3. Results and discussion

3.1 Optimization of LC-MS/MS conditions

First, for mass spectrometry, the electrospray ionization source (ESI) was selected for sample atomization. In order to set the ESI-MS conditions, full scan mass

spectrums and full scan production spectrums of FY363, gemcitabine and IS were investigated in both positive and negative ion mode. The signal intensities found in the positive ionization mode were much more higher compared with the negative mode for all of the analytes and IS, because of alkyl amine groups in their structures. All analytes and IS predominantly formed protonated molecules of $[M+H]^+$ in the full scan spectrum. No sodium or other solvent adducts or dimmers were observed. Figure 2 shows the product ion spectra of FY363, gemcitabine and IS. The major fragment ions were obtained at m/z 212.1, 112.2 and 179.8 for FY363, gemcitabine and IS, respectively. The maximum intensity of mass spectral signals was obtained by selecting these major fragment ions.

Second, for chromatographic separation, the big difference in polarity between FY363 and gemcitabine makes it challenged to elute these two compounds in a single run. With an ammonia group in cytosine ring, gemcitabine is much more hydrophilic than FY363 and has a very short retention time when separated using reversed phase chromatography on ordinary C18 column. To overcome this problem, a novel column of Synergi 4u Polar-RP 80A (Phenomenex, Torrance, CA, USA) which has a unique ether - phenyl bonded phase and a polar group capped was identified as the best choice. This highly stable polar column could have the greatest retention and selectivity for polar or aromatic compounds. Both gemcitabine and FY363 were appropriately retained on this selected column. Besides, a gradient elution mode was optimized to achieve complete separation and nice peak shape in order to avoid interference between the two analytes or between the IS and the analyte. A mixed solvent containing methanol-acetonitrile (3:2, v/v) was used as organic modifier because acetonitrile eluted gemcitabine too rapidly while methanol eluted FY363 too slowly.

3.2 Optimization of sample preparation

LY2334737, one gemcitabine amide derivative of FY363 analog, has been reported to be hydrolyzed into gemcitabine by carboxylesterase (CES) in the body [28, 30]. Similar situation is likely to happen on FY363. Due to the higher plasma CES

activity in rat and mice than in other animals [34-36], FY363 in rat plasma may hydrolytically degrade before sample preparation, affecting the accuracy of the FY363 assay in naive plasma. To avoid this problem, plasma samples were pretreated by the addition of SDS [37] to inactivate CES activity when sampling. In order to optimize the amount of SDS used, 1mL of rat heparinized whole blood was added with 50μL of different concentration of SDS (0.1, 0.2, 0.4 g/ml), respectively. A negative control was carried out without SDS added in blood. Whole blood was centrifuged for 5 min at 8000 rpm to prepare plasma. 45μL of each pre-treated plasma was spiked with 5μL of FY363 to give a final concentration of 2000 ng/mL. Plasma samples were then incubated at 37℃ for 0h, 2h and 6h, respectively. After incubation, the samples were analyzed. Fig.4A shows the stability of FY363 in the samples prepared without SDS or with SDS. In the plasma samples pre-treated without SDS and incubated at 37℃ for 2h and 6h, the amounts of FY363 were decreased to 87.4 and 65.6% of those at the start of incubation, respectively, and that of gemcitabine was increased significantly. In contrast, in the samples pre-treated with SDS, no reduction in FY363 and no production in gemcitabine were observed regardless of SDS concentration.

Another related problem is the stability of gemcitabine in plasma, generally it is accepted that the addition of tetrahydrouridine (a cytidine deaminase inhibitor) prevents metabolism of gemcitabine in the plasma samples during extraction giving a reliable results[38-40]. Therefore, we investigated if SDS was going to provide this protection. Fig.4B shows the stability of gemcitabine in the samples prepared without SDS or with SDS. The method of preparing the sample was exactly the same as that of the FY363 stability study. In the plasma samples pre-treated without SDS and incubated at 37℃ for 2h and 6h, the amounts of gemcitabin were decreased to 90.1 and 82.7% of those at the start of incubation, respectively. However, there was almost no degradation (<3%) of gemcitabine after SDS was added, even with the lowest SDS concentration. Based on the stability results of FY363 and GT in rat plasma, a relatively low concentration of SDS (0.1g/ml) was finally selected as stabilizer when blood sampling.

In most cases, due to the difficulty of extraction, protein precipitation (PPT) is

the preferred choice for polar compounds such as gemcitabine. However, PPT is usually the least efficient in sample cleaning since there are many residual matrix components that can cause undesirable ion suppression for LC-MSMS and contaminate the ion source [41]. Therefore, in this manuscript, liquid-liquid extraction (LLE) by ethyl acetate was selected mainly to avoid the potential ion suppression introduced by SDS. Ethyl acetate has a moderate lipophilicity and hydrophilicity, which is suitable to extract FY363 and gemcitabine simultaneously from the plasma matrix. Although the recovery rate of gemcitabine was relatively inferior (about 33%), the LLOQ of gemcitabine (1 ng/ml) is sufficient for the next pharmacokinetic studies of FY363 and its metabolite gemcitabine. Moreover, the sensitivity established for gemcitabine in this manuscript has been much more better than most previously described methods (LLOQs among 5 ng/ml to 200ng/ml in biological matrix) [42-47].

3.2 Method validation

3.2.1 Specificity

Specificity of the method was demonstrated by comparing the chromatograms of blank plasma and plasma containing FY363 and gemcitabine. The typical chromatograms were exhibited in Fig.3. Retention times of FY363, gemcitabine and IS were 7.6, 3.9 and 6.9 min, respectively. Under the established chromatographic conditions, no endogenous plasma components or other impurities interferences were observed at the peak region of FY363, gemcitabine and IS. Both analyte and IS peak shapes were symmetrical. These observations show that the specificity of the method was acceptable.

3.2.2 Linearity and sensitivity

A calibration curve was constructed using a weighted (1/x) least squares linear regression by plotting the peak area ratios of analyte/IS versus nominated plasma concentrations. The calibration curve exhibited a good linearity over a concentration range of 1-1000 ng/mL for both FY363 and gemcitabine. The linear regression equations were y=0.00452x - 0.00221 for FY363 and y=0.00306x - 0.000848 for gemcitabine, respectively.

The correlation coefficient values were 0.9987 for FY363 and 0.9991 for gemcitabine, respectively. The LLOQ both for FY363 and gemcitabine was 1 ng/mL. At this LLOQ, signal noise ratio was greater than 15, and the accuracy and precision were within 9.0% and 5.7%, respectively.

3.2.3 Precision and accuracy

The intra-day and inter-day accuracy, which were expressed as the percentage error, were calculated by comparing the average measured values and the nominal values. The intra-day and inter-day precision were assessed by calculating the relative standard deviation. The accuracy and precision data for FY363 and gemcitabine at three concentration levels were presented in Table 1. The intra- and inter-day precision in rat plasma was between 2.4 and 7.2% for all concentrations in terms of the RSD, and the accuracy was between 0.8 and 9.9% for FY363 and between -5.3 and 4.8% for gemcitabine in terms of the RE. All RE (%) and RSD (%) values met the acceptance limits established by FDA guidance for the validation of bioanalytical methods (2014).

3.2.4 Recovery and matrix effect

The results of the recovery and matrix effect of FY363 and gemcitabine in rat plasma were summarized in Table 2. The mean recovery of FY363 was ranged from 92.8% to 98.3% and the matrix effect ranged from 101.4% to 109.5%. The mean recovery of gemcitabine was ranged from 32.4% to 33.3% and the matrix effect ranged from 91.0% to 93.9%. At three investigated concentration levels of FY363 and gemcitabine, no apparent ionization interference was found and recovery rates were acceptable.

3.2.5 Stability studies

The stability of FY363 and gemcitabine in rat plasma under various storage conditions, including room temperature for 6 h, frozen at -80℃ for 7 days, three freeze-thaw cycles and the post-preparative stability (autosampler stability) at 4℃ for 24h were evaluated at low, medium and high concentrations (Table 3). All RE (%) and

RSD (%) values were below 10%, indicating that FY363 and gemcitabine were stable under typical sample storage and processing conditions.

3.3 Pharmacokinetic study in SD rats

The validated LC-ESI-MS/MS method was successfully applied to a pharmacokinetic study of FY363 in rats after intravenous administration of FY363 and its active metabolite gemcitabine. The mean plasma concentration-time profiles of FY363 and gemcitabine after administration were shown in Fig. 5. The pharmacokinetic parameters based on non-compartmental method were summarized in Table 4. The rate constant (K_{01}) of gemcitabine after intravenous administration of FY363 was calculated based on one-compartment model of oral administration. K_{01} represents the rate of absorption in non-intravenous administration model. The conversion time of FY363 hydrolysis into gemcitabine in vivo was obtained by K_{01} reciprocal. Equal molar doses of FY363 and gemcitabine were administered to the rats to understand the metabolic fraction of FY363 converted to gemcitabine.

After FY363 intravenous injection, the plasma concentration of FY363 prototype continued to decline (see Fig.5A), the initial plasma concentration (C_0) of FY363 was about 63.92 μmol/L, and the plasma exposure ($AUC_{0-\infty}$) of FY363 was about 24.58 h*μmol/L. However, as for the gemcitabine, an active metabolite after intravenous administration of FY363, its plasma concentration first increased and then decreased (see Fig.5B), the maximum plasma concentration (C_{max}, about 1.72 μmol/L) appeared at about 1.00 h (T_{max}), and the mean plasma exposure was about 14.02 h*μmol/L. In addition, as calculated from K_{01}, the conversion time of FY363 to gemcitabine was about 12.61 min, indicating that FY363 degraded rapidly to generate gemcitabine in vivo after intravenous administration. However, the exposure amount of FY363 prototype in vivo was about twice as much as its metabolite gemcitabine, indicating that FY363 prototype was still the predominant form in vivo.

After direct intravenous administration of gemcitabine at equimolar dose, the plasma concentration of gemcitabine continued to decline (see Fig.5C), the initial plasma concentration of gemcitabine was about 27.20 μmol/L, and the mean plasma

exposure of gemcitabine was about 71.28 h*μmol/L. By comparing the exposure (AUC) of gemcitabine after the FY363 administration with that after direct gemcitabine administration at equimolar dose, the metabolic fraction of FY363 converted to gemcitabine was about 20% in rats. In addition, the elimination of gemcitabine was significantly slowed down after FY363 administration compared to the direct administration of gemcitabine, resulting in the elimination half-life ($t_{1/2}$) of gemcitabine prolonged nearly 3-fold (see Table 4). A gradual release of gemcitabine following cleavage of the carbamate bond in FY363 would enhance efficacy, since more cancer cells would be exposed to a continuous effective cytotoxic level of gemcitabine.

3. Conclusion

A novel and specific LC-MS/MS method was developed for the simultaneous determination of FY363 and gemcitabine in rat plasma for the pharmacokinetic evaluation of FY363 in rats. A simple liquid–liquid extraction method was applied and this method guaranteed excellent sensitivity, precision, accuracy, selectivity, recovery and reproducibility. Moreover, this is the first report to demonstrate the pharmacokinetic behavior and the metabolic transformation of FY363 in vivo. In particular, FY363 could effectively prolong the exposure of gemcitabine in vivo. All these would certainly provide valuable information for the further development of FY363.

Conflict of interest

The authors declare that they have no conflict of interest.

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Tables

Table 1 The intra-day and inter-day precision and accuracy of FY363 and gemcitabine (n = 6).

Analyte	Spiked concentration (ng/ml)	Intra-day			Inter-day		
		Measured concentration (ng/ml)	Precision (RSD%)	Accuracy (RE%)	Measured concentration (ng/ml)	Precision (RSD%)	Accuracy (RE%)
FY363	3	3.3 ± 0.1	2.5	9.9	3.1 ± 0.2	6.9	3.5
	50	53.3 ± 2.4	4.5	6.5	50.4 ± 3.2	6.4	0.8
	1000	1046.7 ± 25.0	2.4	4.7	1007.8 ± 48.2	4.8	0.8
Gemcitabine	3	3.0 ± 0.1	2.4	1.1	3.1 ± 0.1	4.0	2.0
	50	50.9 ± 3.6	7.2	1.7	52.4 ± 2.8	5.3	4.8
	1000	947.5 ± 32.2	3.4	-5.3	987.5 ± 54.7	5.5	-1.2

RSD means the relative standard deviation, RE means the relative error.

Table 2 Extraction recovery and matrix effect data for FY363 and gemcitabine (n=6).

Analyte	Spiked concentration (ng/mL)	Recovery (%)	Matrix effect (%)
FY363	3	92.8 ± 10.1	109.5 ± 8.1
	50	98.3 ± 4.9	101.4 ± 7.1
	1000	95.8 ± 8.0	103.8 ± 5.0
Gemcitabine	3	32.4 ± 3.2	91.0 ± 10.4
	50	33.0 ± 2.0	93.9 ± 5.8
	1000	33.3 ± 2.3	93.4 ± 5.9

Table 3 Stability of FY363 and gemcitabine in rat plasma under different storage conditions (n = 6).

Storage conditions	Spiked concentration (ng/mL)	FY363			Gemcitabine		
		Measured concentration (ng/ml)	Precision (RSD%)	Accuracy (RE%)	Measured concentration (ng/ml)	Precision (RSD%)	Accuracy (RE%)
Room temperature for 6 h	3	3.2 ± 0.1	4.3	5.5	2.9 ± 0.1	4.0	-3.3
	50	54.3 ± 1.7	3.2	8.5	48.5 ± 3.3	6.8	-2.9
	1000	1083.3 ± 37.2	3.4	8.3	931.5 ± 32.7	3.5	-6.9
Frozen (-80℃) for 7 days	3	3.2 ± 0.1	3.5	7.2	2.8 ± 0.1	4.3	-5.8
	50	52.2 ± 1.2	2.3	4.5	47.4 ± 1.6	3.4	-5.2
	1000	1056.7 ± 33.3	3.1	5.7	937.0 ± 21.1	2.2	-6.3
Three freeze-thaw cycles	3	3.3 ± 0.1	3.0	8.3	2.8 ± 0.1	4.8	-6.2
	50	53.6 ± 1.8	3.4	7.1	47.9 ± 3.0	6.3	-4.2
	1000	1071.7 ± 26.4	2.5	7.2	962.3 ± 45.3	4.7	-3.8
Autosampler stability (24 h at 4℃)	3	3.2 ± 0.1	3.2	7.6	2.9 ± 0.2	6.5	-4.6
	50	49.2 ± 1.3	2.6	-1.6	47.2 ± 1.0	2.1	-5.6
	1000	1086.7 ± 50.5	4.6	8.7	947.0 ± 57.3	6.1	-5.3

Table 4 Pharmacokinetics parameters after intravenous administration (i.v.) of FY363 and gemcitabine at equimolar dose (50 μmol/kg) to rats.

Parameters	i.v. FY363		i.v. Gemcitabine
	FY363 (Prototype)	Gemcitabine (Metabolite)	Gemcitabine (Prototype)
C_0 (μmol/L)	63.92 ± 6.43	-	27.20 ± 3.84
C_{max} (μmol/L)	-	1.72 ± 0.12	-
AUC_{0-24h} (h*μmol/L)	24.57 ± 0.94	13.43 ± 1.26	70.38 ± 10.61
$AUC_{0-\infty}$ (h*μmol/L)	24.58 ± 0.94	14.02 ± 1.22	71.28 ± 10.66
$t_{1/2}$ (h)	1.52 ± 0.06	5.52 ± 0.47	1.85 ± 0.11
MRT (h)	1.80 ± 0.14	7.37 ± 0.35	2.67 ± 0.16
V or V/F (L/kg)	4.51 ± 0.36	-	2.14 ± 0.41
CL or CL/F (L/h/kg)	2.05 ± 0.08	-	0.80 ± 0.14
T_{max} (h)	-	1.00 ± 0.22	-
K_{01} (1/h)	-	5.21 ± 0.80	-

Figure legends

Fig.1 Structures of Gemcitabine (a) and the related nucleoside prodrugs such as LY2334737 (b), Capecitabine (c) and FY363 (d).

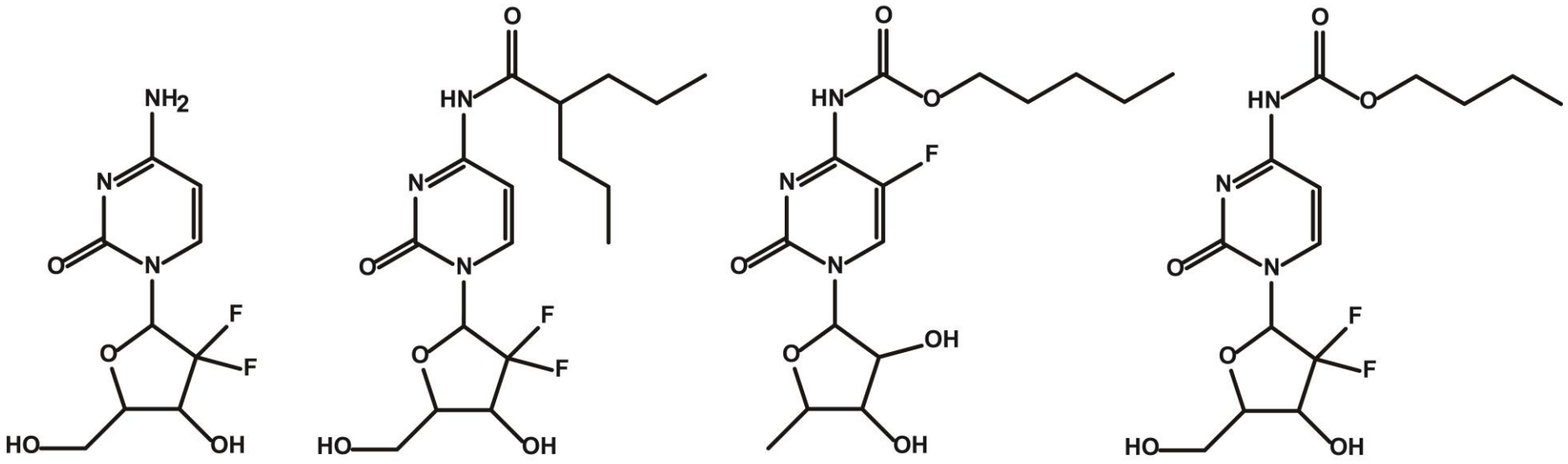
Fig.2 Product ion mass spectra of (a) FY363 (m/z 364.0→212.1), scan range 50-400 amu; (b) Gemcitabine (m/z 264.0→112.2), scan range 50-300 amu and (c) IS (Saxagliptin) (m/z 316.2→179.8), scan range 50-350 amu in the positive ionization mode.

Fig.3 Multiple reaction monitoring ion-chromatograms of (A) blank plasma; (B) blank plasma spiked with FY363 (30 ng/mL in plamsa) and extracted by blank ethyl acetate; (C) blank plasma spiked with gemcitabine (30 ng/mL in plamsa) and extracted by blank ethyl acetate; (D) blank plasma extracted by ethyl acetate containing 50 ng/ml IS (saxagliptin); (E) blank plasma spiked with mixed standard solution of FY363 and gemcitabine (100 ng/mL in plasma) while extraction by ethyl acetate containing 50 ng/ml IS; (F) rat plasma sample at 2 h after intravenous administration of FY363 (50 μmol/kg).

Fig.4 Stability of FY363 (A) and gemcitabine (B) in the rat plasma pre-treated without SDS (0) or with SDS of different concentrations (0.1, 0.2, 0.4 g/ml). Each pre-treated plasma was incubated at 37℃ for 2h (■) and 6h (□), respectively, and the plasma concentration of FY363 or gemcitabine after the start of incubation was analyzed. The data are expressed as the percentages of the control amount remaining of FY363 or gemcitabine (mean ± SD, n=3) .

Fig.5 Mean plasma concentration-time profiles of (A) FY363 after i.v. dose of 50 μmol/kg FY363; (B) gemcitabine after i.v. dose of 50 μmol/kg FY363; (C) gemcitabine after i.v. dose of 50 μmol/kg gemcitabine to rats, respectively. The inserted represented for the semi-log graph. (mean ± SD, n=6).

Fig. 1



(a) Gemcitabine (b) LY2334737 (c) Capecitabine (d) FY363

Fig. 2

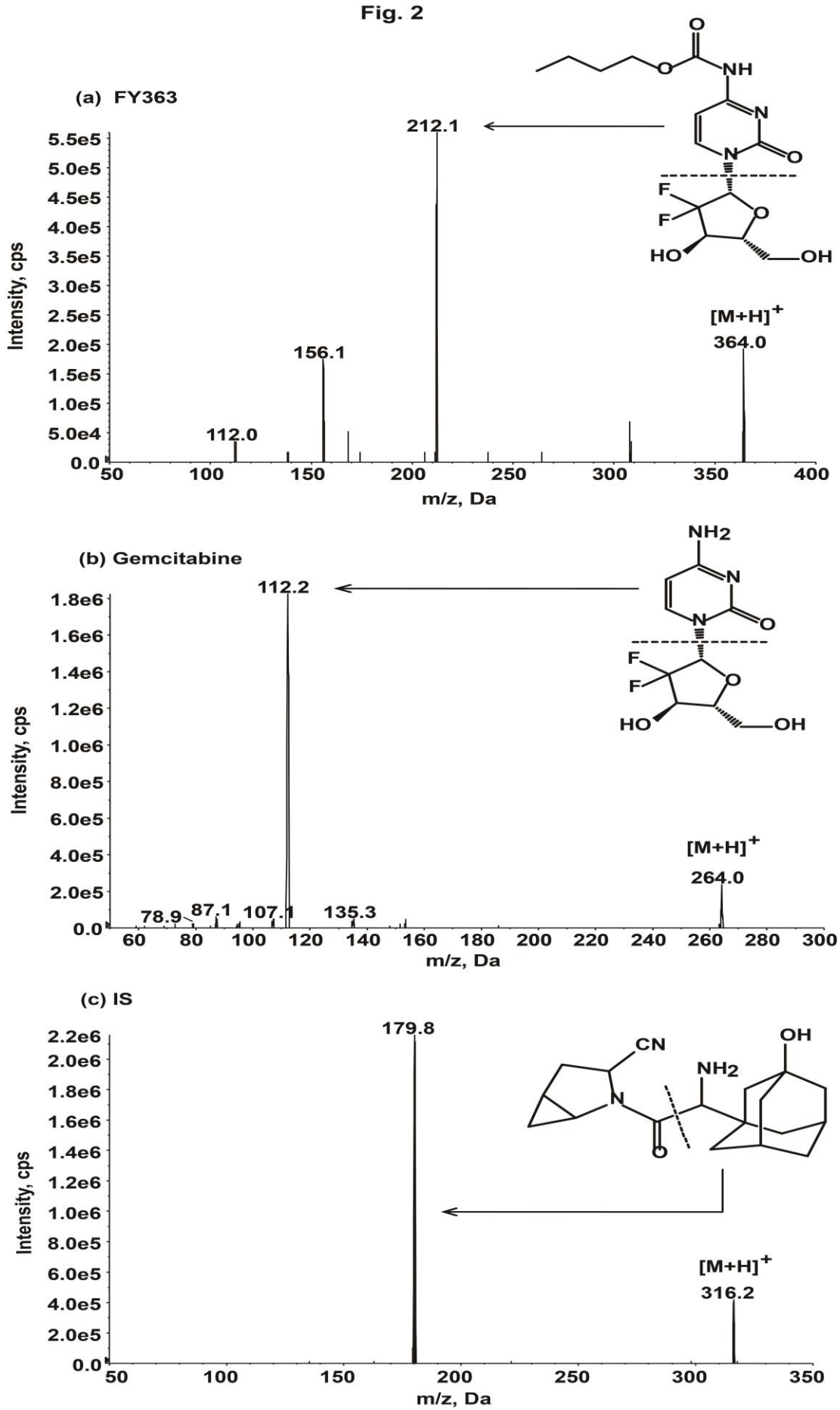


Fig. 3

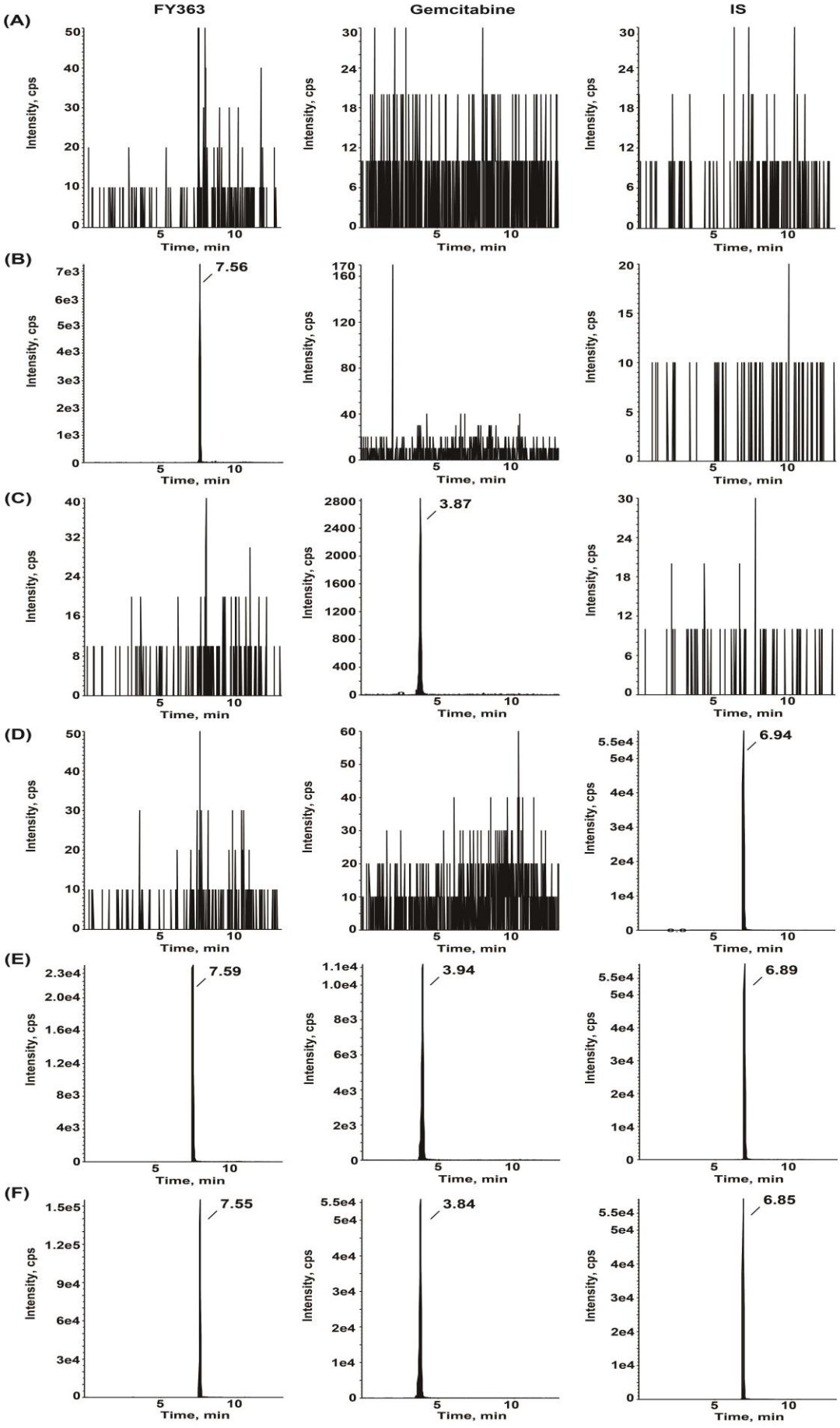


Fig.4

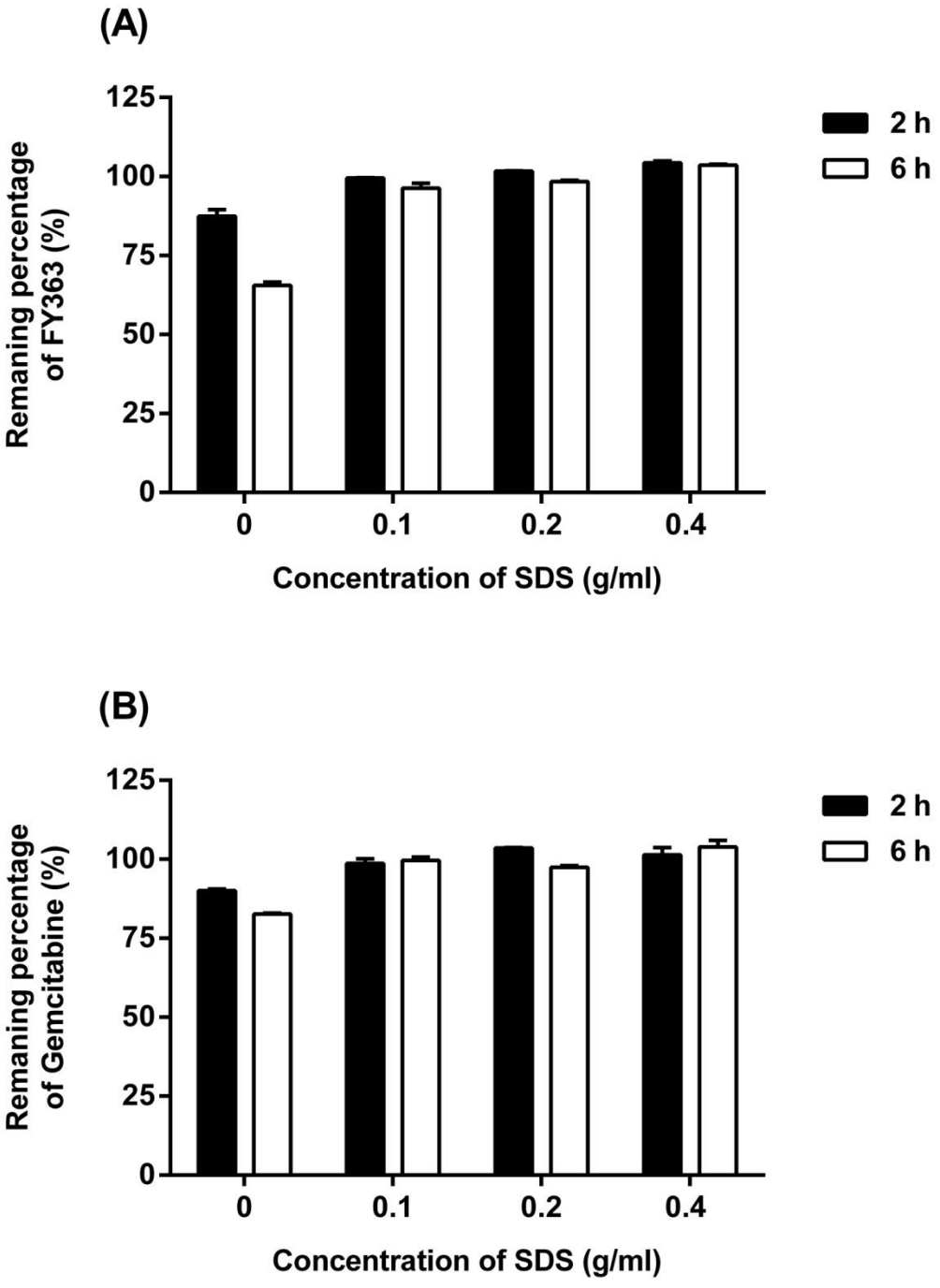
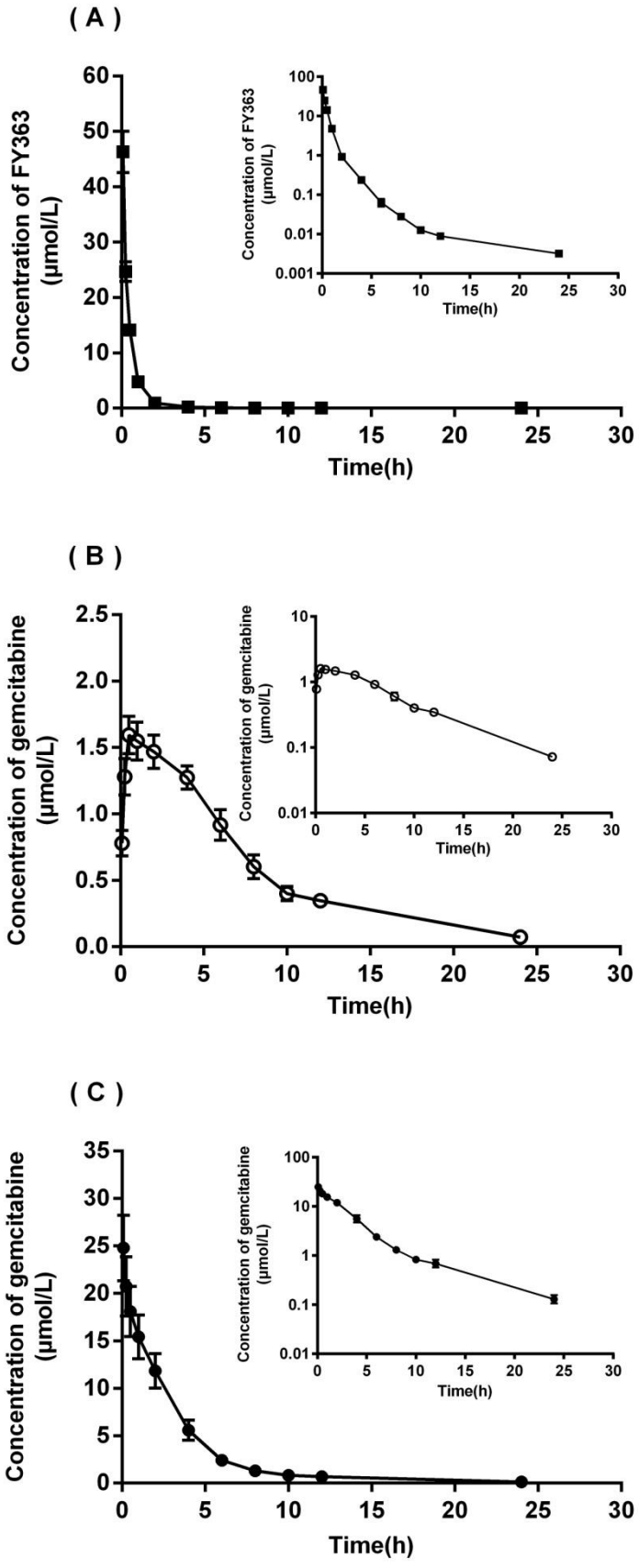


Fig. 5



Highlights

- A novel gemcitabine carbamate prodrug with broad spectrum antitumor activity
- Chromatography on a unique ether - phenyl bonded phase.
- Simultaneous determination of prodrug and gemcitabine in rat plasma.
- About 20% of prodrug converted into gemcitabin in vivo
- This prodrug effectively prolong gemcitabine exposure in vivo.