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A novel brain targeted 5-FU derivative with potential antitumor efficiency and decreased acute toxicity: synthesis, *in vitro* and *in vivo* evaluation

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The broad-spectrum antitumor agent 5-fluorouracil (5-FU), has been used to treat various solid malignant tumors. However, its short life-time *in vivo* and poor ability to cross the blood-brain barrier has limited its application to brain tumor therapy. In order to develop a 5-FU derivative that localizes efficiently to the brain while retaining potent antitumor activity, we conjugated 5-FU with *N,N*-dimethylethylenediamine *via* an amide bond. The stability of the resulting 5-FU derivative (D-FU) was tested *in vitro* in phosphate buffer, rat plasma and brain homogenate. The pharmacokinetic and biodistribution studies in brains of the rats showed a higher C_{max} (the maximal concentration) and an increased AUC_{0-t} (the area under the concentration-time curve) which was 6-fold that of 5-FU. In addition, compared to 5-FU, D-FU exhibited lower toxicity in an acute toxicity assay and similar antitumor activity in the C6 cell line. In conclusion, D-FU has the potential to be developed into an efficient brain delivery drug.

1. Introduction

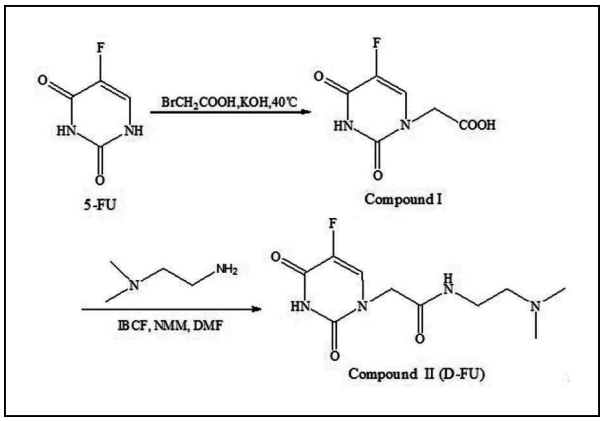
The most challenging task when treating nervous system diseases is delivering drugs to the brain: the blood–brain barrier (BBB) excludes nearly 98% of small molecules and 100% of large molecules (Chen et al. 2004; Pardridge 2005). This limits the efficiency of pharmacological treatments for many diseases of the central nervous system (CNS), even when the same drugs effectively treat the corresponding disease outside the CNS. For example, 5-fluorouracil (5-FU) is widely used to treat various solid malignant tumors such as colorectal cancers (Daumar et al. 2011). However, it works poorly against brain tumors, largely because it crosses the BBB extremely inefficiently (Brem and Lawson 1999). In fact, the lack of uniformly successful drug treatments for malignant brain tumors mean that they still cause approximately12690 deaths in the United States every year (Gududuru et al. 2004; Garcia et al. 2007).

5-Fluorouracil, rationally designed more than 50 years ago (Heidelberger et al. 1957), is an antimetabolite drug that inhibits the synthesis of DNA and RNA during the S-phase of the cell cycle (Scherf et al. 2000). Despite its widespread use to treat tumors, it presents several disadvantages (Grem 1996; Daher et al. 1990). One is short lifetime *in vivo*: 80% of the administered 5-FU is catabolized into inactive metabolites (Grem 1996; Daher et al. 1990). Another advantage, the high toxicity, also limits the clinical use of 5-FU (Di Paolo et al. 2001).

Investigators have adopted various approaches in order to deliver 5-FU efficiently to the brain. Fournier et al. (2003, 2004) formulated microspheres based on poly (methylidene malonate 2.1.2) (short for PMM 2.1.2), which they loaded with 5-FU. Local, stereotactic injection into a rat model of brain cancer induced by C6 cell line had inhibited the growth of brain tumor cells. This approach may be difficult to implement widely in the clinic because stereotactic delivery directly into the tumor site requires highly trained and highly skilled personnel (Malakoutikhah et al. 2011). Some researchers developeda transferrin-coupled liposomal system (Soni et al. 2005), in which 5-FU was encapsulated into liposomes carrying transferrin, which allowed the liposomes to enter the brain *via* transferrin receptor-mediated transcytosis (Qian et al. 2002; Cheng et al. 2004). In a third approach, researchers encapsulated the 5-FU prodrug *N*-hexylcarbamoyl-5-fluorouracil into surface-modified nanogels to deliver 5-FU to brain tissue through the intravenous route. Though vesicular vectors can transport 5-FU across the BBB, the uptake by the reticuloendothelial system (RES), especially by the macrophages of liver and spleen, resulted to a significant accumulation of 5-FU in these organs and may lead to unexpected toxicities (Soni et al. 2006).

An approach that may avoid all of these drawbacks, but which to our knowledge has not yet been attempted, would be to conjugate 5-FU with a low molecular weight moiety that would efficiently direct the derivative into the brain. In previous work, our group has shown that *N,N*-dimethylethylenediamine related structures significantly enhance the efficiency by which naproxen and dexibuprofen accumulate in the brain (Zhang et al. 2012a,b). This ligand offers several advantages for drug development, including a simple, small, well-defined structure and apparent lack of toxicity, which contrasts to the toxic effects sometimes reported with nano-particle vectors.

Therefore we sought to create a safe and effective antitumor derivative of 5-FU that targets the brain. We conjugated *N,N*-dimethylethylenediamine with 5-FU *via* an amide bond, and we confirmed the structure of the resulting D-FU conjugate using NMR and ESI-MS spectroscopy. In a series of *in vitro*



Scheme: Synthesis of D-FU.

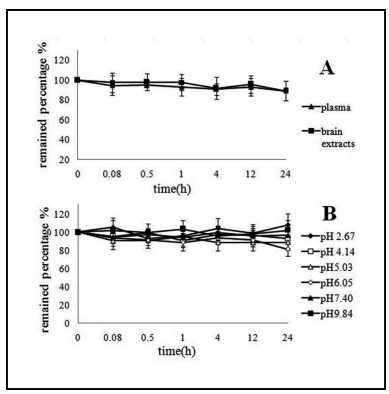


Fig. 1: Stability of D-FU (A) rat plasma and ratbrain extracts, and in (B) phosphate-buffered solutions at different pH values. Data are the mean ± SD (n = 3).

experiments, we examined the stability of D-FU in phosphate buffers with different pH values, as well as in rat plasma and brain homogenate. We also examined the antitumor activity of D-FU in a C6 rat glioma cell line. In a series of *in vivo* experiments, we conducted biodistribution and pharmacokinetic studies in rats to assess the ability of D-FU to target the brain. Furthermore, the acute toxicology study was conducted to evaluate the safety of D-FU.

2. Investigations and results

2.1. Synthesis and in vitro stability study of D-FU

D-FU was synthesized as described in the Scheme, and the structure was confirmed by ^{1}H NMR, ^{13}C NMR as well as ESI-MS. The stability of D-FU, especially in blood and in brain tissue was tested by incubating with phosphate buffer solutions of different pH values ranging from 2.5 to 9.0, as well as in rat plasma and in rat brain homogenate. After 24 h incubation at 37 °C, 80–110% of the original amount of D-FU remained in the buffered solutions (Fig. 1A), while > 90% remained in plasma and brain homogenate (Fig. 1B). These results indicated that D-FU was stable under physiological conditions, opening the door to further studies in cell culture and animals.

2.2. Pharmacokinetic study

We administered D-FU or 5-FU intravenously to rats and measured the concentrations of the drugs in plasma at different time points in order to calculate the pharmacokinetic parameters (Table 1). The concentration of D-FU in plasma was significantly higher than that of 5-FU throughout the time course (Fig. 2). At

Table 1: Pharmacokinetic parameters of 5-FU and D-FU in plasma after i.v. administration to rats

Parameters	5-FU	D-FU
AUC_{0-t} (μmol/g·min)	18.547 ± 0.351	80.323 ± 5.441**
C_{max} (mmol/L)	0.374 ± 0.0342	0.979 ± 0.068**
T_{max} (min)	2	2
$t_{1/2z}$ (min)	112.739	318.132
MRT (min)	22.838 ± 6.823	86.038 ± 9.763**
CLz (L/min/kg)	0.043 ± 0.0152	0.008 ± 0.001**

The concentration of D-FU was converted to 5-FU equivalent. Data represent the mean ± SD ($n = 5$). (** $p < 0.01$). AUC_{0-t}: The area under the concentration-time curve; C_{max}: the maximal concentration; $t_{1/2z}$: biological half life; MRT: the mean residence time; CLz: clearance

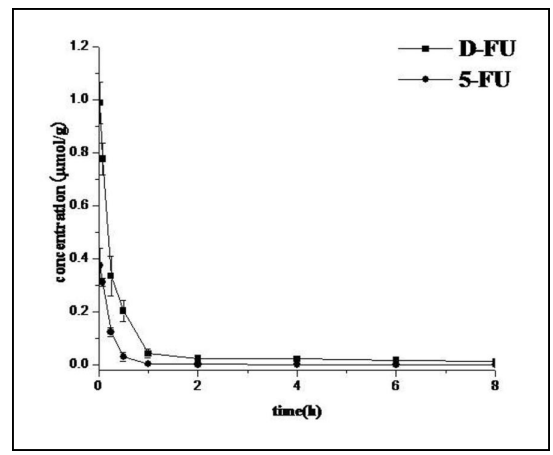


Fig. 2: Concentration of D-FU and 5-FU in plasma at different times after intravenous administration in rats. Data are the mean ± SD (n = 5).

2 min after administration, the mean concentration of D-FU in plasma was 2.6 folds higher than the value for 5-FU. In addition, the area under the curve (AUC_{0-t}) of the test group was 4.3 folds higher compared to the free 5-FU group. These results suggested that the D-FU has a longer half-life and higher AUC_{0-t} in plasma, indicating that D-FU may have higher bio availability and more chance to exhibit antitumor effect.

2.3. Biodistribution study

In order to evaluate the ability of D-FU to target the brain, we analyzed the concentration of D-FU in different tissues at different times after intravenous injection into rats. In parallel, we performed the same experiment with the equivalent dose of 5-FU. Both drugs showed the highest concentrations in most organs within a few minutes of administration, after which their concentrations rapidly decreased (Fig. 3). Comparison of the drug concentrations specifically in brain tissue showed that D-FU showed much greater ability to target the braint issue

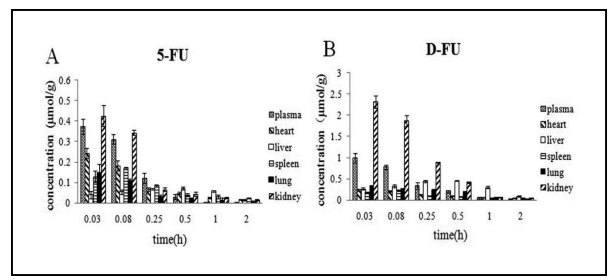


Fig. 3: Biodistribution of (A) 5-FU and (B) D-FU at different times after intravenous administration in rats. Data are the mean ± SD (n = 5).

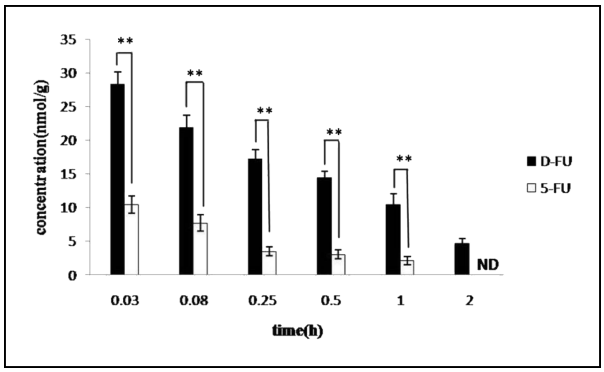


Fig. 4: Concentrations of D-FU (black) and 5-FU (white) in rat brain at different times after intravenous administration. Data are the mean ± SD (n = 5). **, p < 0.01.

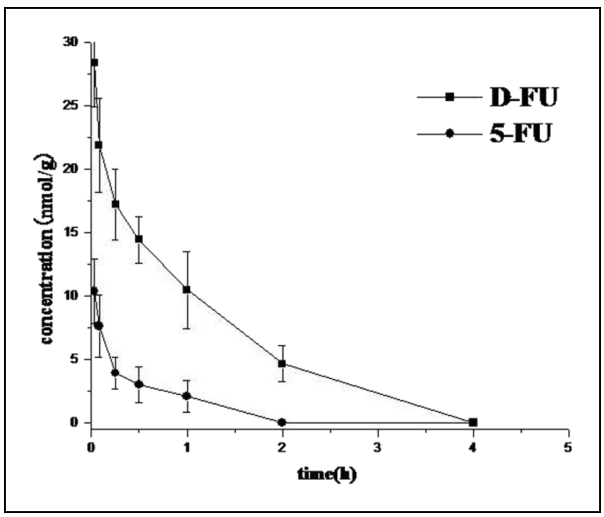


Fig. 5: Concentrations of D-FU (squares) and 5-FU (diamonds) in rat brain at different times after intravenous administration. Data are the mean ± SD (n = 5).

than 5-FU did (Fig. 4). The concentration of D-FU in brain was much higher than that of 5-FU throughout the time course (Fig. 5). Analysis of the pharmacokinetic parameters of both drugs in the brain (Table 2) indicated that $AUC_{0\rightarrow t}$ of D-FU was 6.02 folds higher than the value for 5-FU. Meanwhile, D-FU had an increased half-life and decreased CLz in brain tissue compared to free 5-FU group, suggesting that the derivative was better retained in brain than free 5-FU.

Table 2: Pharmacokinetic parameters of 5-FU and D-FU in brain after i.v. administration

Parameters	5-FU	D-FU
AUC_{0-t} (μmol/g·min)	0.229 ± 0.024	1.3912 ± 0.144**
Cmax (nmol/g)	10.385 ± 1.046	28.372 ± 3.349**
Tmax (min)	2	2
$t_{1/2z}$ (min)	52.494	60.36
MRT (min)	21.117 ± 2.321	42.267 ± 3.987**
CLz (L/min/kg)	1.964 ± 0.185	0.468 ± 0.127**
RE	–	6.02
CE	–	2.7

The concentration of D-FU was converted to 5-FU equivalent. Data represent the mean ± SD ($n = 5$). (**$p < 0.01$ with respect to the corresponding value for 5-FU). RE: the relative uptake efficiency; CE: concentration efficiency.

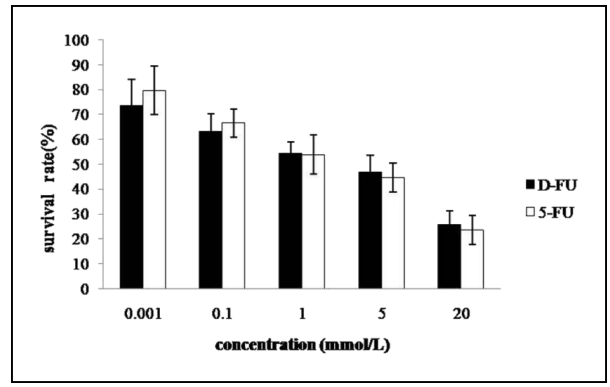


Fig. 6: Cell survival rate of D-FU (black) and 5-FU (white) in C6 cell line by MTT assay. Data are the mean ± SD (n = 3).

2.4. Evaluation of antitumor activity in vitro

The fact that D-FU appeared to be stable *in vivo* and to target the brain efficiently led us to examine its antitumor activity. We chose the C6 rat glioma cell line, which is routinely used to test antitumor toxicity of numerous compounds, including 5-FU (Pang et al. 2010; Fournier et al. 2003). Both D-FU and 5-FU showed concentration-dependent toxicity in C6 cell line in the MTT assay over the concentration range of 0.001–20mmol/L (Fig. 6). Although the stability assay suggested that D-FU did not spontaneously dissociate to release free 5-FU to a significant extent over 24 h, equivalent concentrations of D-FU and 5-FU provoked similar extents of cell death within 24 h ($P > 0.05$), demonstrating that D-FU had kept the antitumor efficiency of 5-FU in C6 cell line.

2.5. Acute toxicity study of D-FU in mice

The acute toxicity study was employed to estimate the safety of D-FU. Mice were administered D-FU or 5-FU and observed for 14 days to detect mortality and any significant changes in behavior compared to their behavior prior to drug administration. Mortality, LD_{50} and behavior were compared between the two groups. The LD_{50} of D-FU was 1210.1 mg/kg (95% confidence interval 1081.3 to 1354.3 mg/kg). In contrast, the LD_{50} of 5-FU was 202.82 mg/kg (95% confidence interval 167.22 to 245.34 mg/kg). These findings suggest that, D-FU retains the antitumor activity of 5-FU, while showing significantly lower acute toxicity.

3. Discussion

Despite its widespread use as an antitumor agent, 5-FU is not routinely used to treat brain tumors because of its poor ability to accumulate in the brain and its high toxicity. Here we show that conjugating 5-FU to *N,N*-dimethylethylenediamine creates a derivative that efficiently targets the brain *in vivo* and shows similar antitumor activity as 5-FU *in vitro,* but with much lower acute toxicity *in vivo.*

In previous work, our research group showed that conjugating *N,N*-dimethylethylenediamine-related structures to the drugs naproxen and dexibuprofen significantly enhanced their concentrations in brain tissue (Zhang et al. 2012a,b). The most efficient one among them was (2*S*)-2-(4-isobutylphenyl) propionic acid 2-dimethylaminoethyl ester (prodrug1) (Zhang et al. 2012b), with a CE value of 7.75. Based on that work, we chose to use *N,N*-dimethylethylenediamine as a ligand to conjugate with 5-FU to target brain tissue. Our biodistribution study showed that the concentration of D-FU in the brain was higher than that of 5-FU over the entire time course examined; the RE

Table 3: Mortality and behavioral changes in mice within 14 days after intravenous administration of different doses of 5-FU

Dose(mg/kg)	Log-transformed dose	Mortality by 14 days	Behavioral changes
103	2.0128	0 of 10	None
147	2.1673	3 of 10	Decreased locomotor activity in some mice.
210	2.3222	5 of 10	Decreased locomotor activity in some mice.
301	2.4786	8 of 10	Loss of body weight and decreased locomotor activity.
430	2.6335	10 of 10	Loss of body weight and decreased locomotor activity. Death was observed from the fourth day after observation

Table 4: Mortality and behavioral changes in mice within 14 days after intravenous administration of different doses of D-FU

Dose (mg/kg)	Log-transformed dose	Mortality by 14 days	Behavioral changes
814	2.9106	0 of 10	None
992	2.9965	3 of 10	None
1210	3.0828	5 of 10	Short ofbreath in some mice
1476	3.1691	7 of 10	Convulsion and breathless in someof mice.
1800	3.2553	10 of 10	Become Wheezing, twitching in some mice, decrease locomotor activity and died that day.

value of D-FU was 6.02 and the CE value was 2.7, indicating efficient transport into the brain. The apparent ability of *N,N*-dimethylethylenediamine to increase D-FU accumulation in the brain is consistent with our earlier work (Zhang et al. 2012b). In fact, various drugs that target the CNS contain structures related to *N,N*-dimethylethylenediamine, such as meclofenoxate, cetirizine diphenhydramine, dextropropoxyphene and procaine (Zhang et al. 2012b).

To ensure a stable linkage between *N,N*-dimethylethylenediamine and 5-FU in our conjugate, we chose an amide bond. Our stability studies in solution and in biological samples indicate that the amide linkage in D-FU is quite stable, suggesting that significant amounts of 5-FU would not be released in the bloodstream, where it could exert toxic effects outside the brain. Pharmacokinetic studies further showed that D-FU accumulates quickly in the brain, suggesting that the effects of the conjugate should be confined to the brain.

Why D-FU targets the brain much more efficiently than does 5-FU is unclear, given that conjugation with *N,N*-dimethylethylenediamine is not expected to increase the inherently low lipophilicity of 5-FU (Zhang et al. 2012b). We speculate that D-FU does not cross the BBB by passive diffusion but rather by an active transport process. Since *N,N*-dimethylethylenediamineis is structurally similar to some organic cations and choline, D-FU may be transported into the brain by transporters that normally carry those ligands (Zhang et al. 2012b).

Our biodistribution and pharmacokinetic studies of D-FU in rats showed that the derivative was present in much higher concentrations than was 5-FU in most organs examined, and that these higher concentrations persisted over several hours. These findings suggest that D-FU is catabolized much more slowly than 5-FU *in vivo*. Dihydropyrimidine dehydrogenase (DPD) catabolizes more than 80% of the administered 5-FU into inactive metabolites, accounting for the low concentration detected in most organs (Heggie et al. 1987). DPD catalyses the conversion of 5-FU to fluoro-5, 6-dihydrouracil (FUH_2), which is the initial and rate-limiting step in the catabolism of 5-FU (Van Kuilenburg 2004). The simplest explanation for the greater and more persistent accumulation of D-FU in organs is that the *N,N*-dimethylethylenediamine creates a great steric hindrance and inhibits the interaction of DPD with the active agents.

Our *in vitro* cytotoxicity experiment with the C6 rat glioma cell line indicated that D-FU retains the antitumor efficacy of 5-FU, despite the apparent stability of the amide linkage in D-FU. These results are consistent with previous report studies in several cell lines showing that modifications to 5-FU at the N1 position of the pyrimidine ring do not decrease, and in fact sometimes increase, the native compound's antitumor activity (Huang et al. 2007; Ouchi et al. 1998; Ohya et al. 1993). This may be because D-FU retains the pharmacophore of 5-FU, namely the F atom and pyrimidine ring (Van Kuilenburg 2004; Daumar et al. 2011). Future studies should verify our antitumor findings *in vivo* before more detailed structure-activity conclusions can be drawn.

Conjugating *N,N*-dimethylethylenediamineto 5-FU not only significantly enhanced the ability of the drug to accumulate in the brain with no loss in antitumor potency, but it also significantly reduces the acute toxicity of the drug *in vitro* (Tables 3, 4). This is consistent with the observation by many researchers that 5-fluorouracil-1-acetic acid (Compound I) can be used to modify 5-FU without increasing the toxic effects (Chung et al. 1991; Zuo et al. 2001; Kang et al. 2002; Yang Z et al. 2000; Daishu et al. 2001). The much lower acute toxicity of D-FU may allow higher doses to be used, which may boost clinical efficacy.

4. Experimental

4.1. Materials

5-FU (>98% pure) was purchased from Nantong General Pharmaceutical Factory (Nantong, China). Methanol (HPLC grade) was purchased from Kemiou (Tianjin, China). All other chemicals and solvents were analytical grade. Thin-layer chromatography (TLC: silica gel GF254) was used to detect the spots by UV radiation. ^{1}H- and ^{13}C- NMR analysis were per formed using D_2O as solvent by AMX-400 Bruker Spectrometer. Chemical shifts were given in ppm (δ). Mass spectroscopy was performed by Agilent 1200 series RRLC system.

4.2. Animals and cells

Wistar rats (male, 220 ± 20 g) and Kunming mice (male, 22 ± 2 g) were provided by the Laboratory Animal Center of Sichuan University (Chengdu, China). All animals were maintained under standard conditions and allowed free access to food and water. The Sichuan University Animal Ethical Experimentation Committee approved the *in vivo* study protocols, which were designed according to the requirements of the National Act on the Use of Experimental Animals (People's Republic of China).

The C6 rat glioma cell line was cultured in RPMI-1640 (Hyclone, USA) media supplemented with 10% fetal calf serum (FMG-Bio, Shanghai, China), 100IU/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2, and the cell medium was changed every other day.

4.3. Synthesis of 5-FU derivative (D-FU)

4.3.1. Compound I

5-FU (3.9 g, 30 mmol) was dissolved in KOH solution (9 ml, 5.7 mol/L), then an aqueous solution of 2-bromoacetic acid (8 ml, 45 mmol) was added dropwise at the temperature of 40 °C. The mixture was stirred for approximately

4 h, until no free 5-FU was detectable by thin-layer chromatography. The solution was then transferred to anice-bath, and the pH was adjusted to 1.0 with hydrochloric acid (10 mol/L) to obtain the crude product. Compound I was collected by filtration, washed with water for 3 times and then dried in 40 °C as 3.95 g white solid with a yield of 70% (Ouyang et al. 2011).
Compound I: ^{1}H-NMR (400 MHz, D_2O): δ8.10–8.08 (d, 1H, J = 6.8 Hz), 4.37 (s, 2H).
ESI-MS (m/z): calcd for 188.11. obsd 187.01 ($[M-H]^+$)

4.3.2. Compound II

Compound I (0.564 g, 3 mmol) was dissolved in dimethylformamide (DMF, 10 ml) in an ice-salt bath. Then *N*-methylmorpholine (NMM, 0.395 ml, 3.6 mmol), isobutyl chloroformate (IBCF, 0.472 ml, 3.6 mmol) and *N,N*-dimethylethylenediamine were added with stirring. The reaction was allowed to proceed for 3 h, after which it was added to ethanol (20 ml) with vigorous agitation. The mixture was filtered under reduced pressure to obtain a white solid which was washed with ethanol to yield 0.464g of the final product corresponding to 42% overall yield over two steps (Ouyang et al. 2011).
Compound II (D-FU):
^{1}H-NMR (400 MHz, D_2O): δ7.84–7.83 (d, 1H, J = 5.6 Hz), 4.55 (s, 2H), 3.70–3.67 (t, 2H, J = 6.0 Hz), 3.37–3.34 (t, 2H, J = 6.0 Hz), 2.95 (s, 6H).
^{13}C-NMR (100 Hz, D_2O): δ172.07(s), 162.20(s), 153.29(s), 143.97∼141.66(d), 133.89∼133.67(d), 58.88(t), 53.27(t), 45.78∼45.24(q), 37.13(s).
ESI-MS (m/z): calcd for $C_{10}H_{15}FN_4O_3$ 258.11. obsd260.00 ($[M+H+1]^+$)

4.4. In vitro stability of D-FU in phosphate buffer and biological sample

The *in vitro* stability of D-FU was investigated in a series of phosphate buffers (pH 2.5, 4.0, 5.0, 6.8, 7.4, 9.0), in rat plasma and in rat brain homogenate (homogenized and diluted with 0.9% physiological saline). D-FU was dissolved in physiological saline (50 μl), and then added to above mediums at a concentration of 30 μg/ml. The mixtures were incubated at 37 °C and the residual concentration of D-FU was determined by HPLC (section 2.5) at the indicated time points.

4.5. HPLC analysis and sample preparation

For determination of 5-FU, plasma or tissue homogenate (200 μl) was mixed with 5-bromouracil solution (20 μl, 50 μg/ml) as the internal standard, followed by ethyl acetate (3 ml). After vigorous vortexing and centrifugation at 13500 rpm for 10 min, the organic layer was collected and evaporated to dryness at 40 °C under air flow. The residue was dissolved in mobile phase (100 μl) and centrifuged again at 13500 rpm for 10 min. An aliquot (20 μl) of supernatant was injected into the HPLC system described below.
For determination of D-FU, phosphate buffer, plasma or tissue homogenate (700 μl) was mixed with 30% (v/v) $HClO_4$ solution (80 μl). After vortexing and centrifugation at 13500 rpm for 10 min, an aliquot (20 μl) of supernatant was injected into the HPLC system described below.
HPLC assay methods were established to determine the concentration of D-FU or 5-FU in biosamples. Analysis was performed using Angilent instrument (Angilent Technologies, American) which consists of a 1260 Quaternary pump, a G1314C 1260 UV detector, and a G1329B 1260 Auto sampler. Separations were carried out using a Kromasil column (150 × 4.6 mm, ODS, 5 μm) with a corresponding guard column (ODS, 5 μm). The detector was set to monitor the signal at 270 nm, and the flow rate was 1.0 ml/min at 35 °C. The mobile phase to detect D-FU was 50mM 1-heptanesulfonic acid and 10 mM sodium 1-heptanesulfonate adjusted to pH 3.4 with phosphoric acid, the retention times was 9.3 min. The mobile phase to detect 5-FU, the mobile phase was buffer which was 50 mM sodium dihydrogen phosphate adjusted to pH 2.5 with phosphoric acid. The retention time was 4.5 min.

4.6. Pharmacokinetic and biodistribution study of 5-FU and D-FU in rats

Male wistar rats (220 ± 20 g) were fasted for 12 h, and assigned randomly into two groups (n = 30 in each group). Each group was treated with a single intravenous injection of either 5-FU or D-FU at an equivalent 5-FU dose of 35 mg/kg in physiological saline. At the indicated time points, blood samples were collected in heparinized tubes, and centrifuged at 5000 rpm/min for 5 min to obtain plasma. The varioust issue samples, including heart, lung, liver, spleen, kidney and brain were harvested and homogenized in two folds volumes of 0.9% physiological saline. All samples were stored at –40 °C until analysis.

4.7. In vitro anticancercytotoxicity study of D-FU

The antitumor activity of D-FU was assessed using the C6 rat glioma cell line. The 3-(4,5-dimethyl-2-tetrazolyl)-2,5-diphenyl-2*H* tetrazolium bromide (MTT) assay was used to determine the number of surviving cells. In brief, cells were seeded in 96-well plate at a density of 7×10^3cells per well in 200 μl culture medium. After 24 h, cells were treated with 200 μl culture medium containing various concentrations of 5-FU or D-FU and incubated another 24 h at 37 °C. Then, MTT solution (20 μl, 5 mg/ml) was added to each well. After incubation for 4 h, the MTT solution was replaced with 200 μl DMSO to dissolve the formazane. The absorption at 570 nm was measured using a Microplate reader (Varioskan Flash; Thermo Fisher Scientific). Each assay was carried out in triplicate and included a negative control (cultures not exposed to 5-FU or D-FU) and a blank (wells containing medium but no cells). The ratio of cell-survival was calculated according to the following equation.

$$\text{Cell survival rate} = ([\text{Abs}])_{\text{sample}} - [\text{Abs}]_{\text{blank}})/ ([\text{Abs}])_{\text{control}} - [\text{Abs}]_{\text{blank}}) \times 100\% \quad (1)$$

4.8. The acute toxicology study of D-FU in mice

Fifty male Kunming mice (22 ± 2 g) were divided randomly into five groups (n = 10 in each group). Each group was given a single dose of 5-FU or D-FU through the tail vein. The class interval of the dose was 0.70 for 5-FU and 0.82 for D-FU. The mice were observed regularly over the next 14 days, and LD_{50} values and 95% confidence intervals (95% CI) were calculated using SPSS assay.

4.9. Data analysis

The pharmacokinetic parameters were calculated for both 5-FU and D-FU using the Data and Statistics software package (DAS, Shanghai, China). To evaluate the ability of D-FU to target the brain, RE and CE were calculated according to defined as follows.

$$RE = (AUC_{0\text{-}t,\text{D-FU}})/(AUC_{0\text{-}t,5\text{-FU}}) \quad (2)$$

$$CE = (C_{max,\text{D-FU}})/(C_{max,5\text{-FU}}) \quad (3)$$

Statistical evaluation was performed using analysis of variance followed by *t*-test. Differences with an associated $p < 0.05$ were considered significant.

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